Implications of a CALM-derived Nuclear Export Signal for

CALM-AF10-mediated Leukemogenesis

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Dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
the Department of Pharmacology and Cancer Biology
in the Graduate School of Duke University

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ABSTRACT

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Abstract

The t(10;11) chromosomal translocation gives rise to the *CALM-AF10* fusion gene and is found in patients with aggressive and difficult-to-treat hematopoietic malignancies. *CALM-AF10*-driven leukemias are characterized by a perturbed epigenetic and transcriptional state. Specifically, the *HOXA* cluster genes are hypermethylated on Histone H3 lysine 79 (H3K79), which corresponds with their transcriptional upregulation. Conversely, *CALM-AF10* cells display global H3K79 hypomethylation. DOT1L, the H3K79 histone methyltransferase, interacts with the OM-LZ domain of AF10, and the AF10 OM-LZ domain has been shown to be necessary and sufficient for CALM-AF10-mediated transformation. These data have suggested a critical role for the AF10-DOT1L interaction in *CALM-AF10* leukemias. However, the mechanism(s) by which DOT1L-mediated epigenetics are perturbed and the precise role of CALM in leukemogenesis have remained unclear.

In this dissertation, we examine the contribution of CALM to CALM-AF10-mediated leukemogenesis. We determine that CALM contains a functional nuclear export signal (NES) that mediates steady-state cytoplasmic localization of CALM-AF10. An NES is a highly conserved leucine-rich amino acid sequence that is recognized by the nuclear export receptor, CRM1. Classically, CRM1 binds to NES-containing proteins and mediates their export from the nucleus to the cytoplasm through the nuclear pore complex. Through structure-function analyses, we determine that the CALM-derived

NES is necessary and sufficient for CALM-AF10-dependent leukemogenesis. In addition, fusions of NES motifs from heterologous proteins (ABL1, Rev, PKIA, and APC) in-frame with AF10 are sufficient to immortalize murine hematopoietic progenitors *in vitro*. From these data, we conclude that a CRM1-dependent NES represents the functional contribution of CALM for CALM-AF10-mediated leukemogenesis.

In the second part of this dissertation, we examine the mechanism(s) by which the CALM NES imparts transformation potential to AF10. We determine that the CALM NES is essential for CALM-AF10-dependent *Hoxa* gene upregulation and aberrant H3K79 methylation. Using co-immunofluorescence microscopy, we observe increased cytoplasmic localization of DOT1L in the presence of CALM-AF10, suggesting that mislocalization of DOT1L may lead to a global loss of H3K79 methylation. In addition to mediating nuclear export, we find that the CALM-CRM1 interaction is critical for targeting CALM-AF10 to the *Hoxa* locus. Inhibition of CRM1 with Leptomycin B prevents transcription of *Hoxa* genes in *CALM-AF10* leukemia cells. These findings uncover a novel mechanism of leukemogenesis mediated by the nuclear export pathway and support further investigation of the utility of CRM1 inhibitors as therapeutic agents for patients with *CALM-AF10* leukemias.

To Auntie Debbie, my constant inspiration

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List of Abbreviations

aa	amino acid
ABL1	Abelson murine leukemia viral oncogene 1 protein
AF10	ALL1-fused gene from chromosome 10 protein
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	Adenomatous polyposis coli protein
B2M	Beta-2-microglobulin protein
bp	base pair
BM	bone marrow
CALM	Clathrin assembly lymphoid myeloid leukemia protein
CCVs	clathrin coated vesicles
ChIP	chromatin immunoprecipitation
CLPs	common lymphoid progenitors
CMPs	common myeloid progenitors
CRM1	Chromosome region maintenance 1 protein
CT	comparative threshold
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium

DNA	Deoxyribonucleic acid
DOT1L	Disruptor of telomeric silencing-1 like protein
EGFR	Epidermal growth factor receptor
FBS	fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase protein
GFP	Green fluorescent protein
GMPs	granulocyte/macrophage lineage-restricted progenitors
H3K4	Histone H3 lysine 4
H3K79	Histone H3 lysine 79
HEK293	human embryonic kidney 293 cells
Hoxa	Homeobox A cluster protein
HPs	hematopoietic precursor cells
HSCs	hematopoietic stem cells
IB	immunoblot
IF	immunofluorescence
IL-3	Interleukin 3
Kb	kilobase
LMB	Leptomycin B
MEF	murine embryonic fibroblasts
MEIS1	Myeloid ecotropic viral integration site 1 protein

MEPs	megakaryocyte/erythrocyte lineage-restricted progenitors
MLL	Mixed-lineage leukemia protein
MPPs	multipotent progenitors
mRNA	messenger ribonucleic acid
NEI	nuclear export inhibitor
NES	nuclear export signal
NLS	nuclear localization sequence
nt	nucleotide
NUP	nucleoporin protein
OM-LZ	octapeptide motif–leucine zipper
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PKIA	Protein kinase inhibitor alpha protein
Rev	Regulator of expression of Virion protein
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
T-ALL	T-cell acute lymphoblastic leukemia

TfR	Transferrin receptor
TSS	transcription start site

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Words truly are not sufficient to express my gratitude toward those that have helped, encouraged, and supported me through the years. Graduate school has been a long journey with many ups and downs, and the following people have been my foundation.

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critical, passionate, hard-working scientist. She has also been a confidant and source of never-ending support. Through heaping amounts of negative data and the rare positive result, Catherine would be the first with whom I would share the news. She was always there to commiserate or to jump for joy. I cannot imagine getting to this point without Catherine's guidance.

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Within months of starting graduate school, I found myself at an animal shelter adopting a little black ball of fur. The little ball quickly grew into the 80 pound Labrador that I call Saturn. It seems silly to acknowledge a dog in my dissertation, but Saturn has been my furry companion from the very beginning. As it was difficult to move so far from my family, Saturn immediately made Durham feel like home. I thank Saturn for her

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1. Introduction

1.1 Normal hematopoiesis and hematological malignancies

1.1.1 Normal hematopoiesis

Blood contains many types of cells, which function to transport oxygen and nutrients, defend against infection, and form clots. All blood cells have limited life spans and are constantly regenerated during the lifetime of an animal. Hematopoiesis is the tightly controlled process of producing blood cells. Blood cells are derived from multipotent hematopoietic stem cells (HSCs), which are defined by the ability to self-renew and to differentiate into all blood cell lineages (**Figure 1**) (Orkin, 2000). During development, hematopoiesis begins in the yolk sac and shifts to the fetal liver at E11.5-E12.5 (Okuda et al., 1996). After birth, the bone marrow becomes the major location of HSCs and site of hematopoiesis (Mikkola and Orkin, 2006).

Early in hematopoiesis, HSCs give rise to multipotent progenitors (MPPs), which can differentiate into lymphoid or myeloid lineage-committed progenitors (**Figure 1**) (Warr et al., 2011). Unlike stem cells, hematopoietic progenitor cells (HPs) have limited self-renewal capability. Lineage-committed progenitors divide rapidly, but only a limited number of times, before they terminally differentiate (Alberts, 2002). Lymphoid progenitor cells give rise to B-, T-, and natural killer cells, while myeloid progenitors give rise to erythrocytes, megakaryocytes, granulocytes, and monocytes (**Figure 1**). Altogether, hematopoiesis involves multi-step progression from HSCs to mature blood

cells through distinct stages of differentiation. Because of this stepwise commitment, the hematopoietic system can be viewed as a hierarchical family tree of cells (**Figure 1**) (Alberts, 2002).

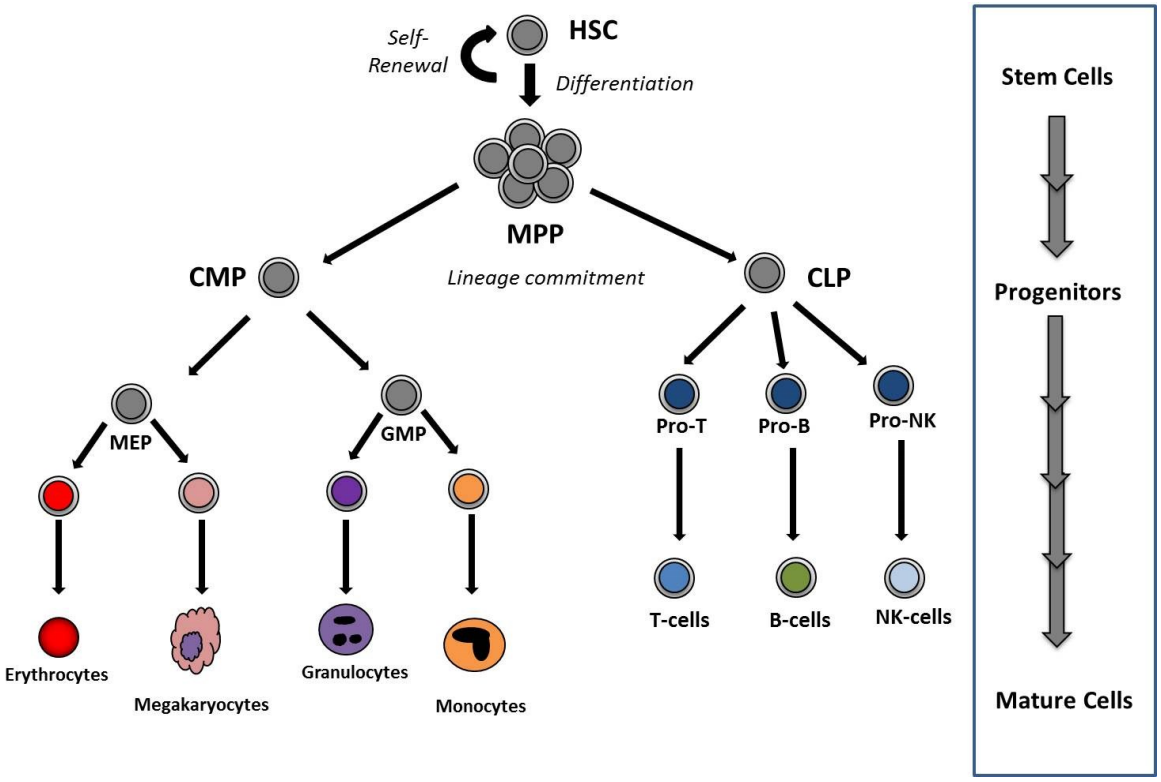

Figure 1: Normal hematopoiesis

Hematopoiesis occurs through a stepwise progression from stem cell to mature blood cell. Hematopoietic stem cells (HSCs) can either self-renew or differentiate into multipotent progenitors (MPPs). MPPs progress to lineage-committed progenitors, common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). CLPs differentiate into mature B-, T- and NK- cells by stepwise progression. CMPs give rise to granulocyte/macrophage lineage-restricted progenitors (GMPs) and megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs). Subsequently, monocytes and granulocytes arise from GMPs and megakaryocytes and erythrocytes arise from MEPs. For simplicity, dendritic cells and further classified white blood cells were not included in this figure. Adapted from (Warr et al., 2011).

During adult life, HSCs are maintained in a quiescent (G0) state of the cell cycle (Pietras et al., 2011). This contributes to their long-lived maintenance yet allows HSCs to quickly re-enter the cell cycle (Warr et al., 2011). Re-entry of HSCs into the cell cycle and subsequent self-renewal or hematopoietic differentiation is dependent on the interplay between intrinsic genetic processes and external factors from the microenvironment or niche (Pietras et al., 2011; Warr et al., 2011; Zon, 2008). Intrinsically, alternate expression of specific transcriptional regulators, growth factors, and growth factor receptors determine lineage commitment and maturation. The Notch, Sonic Hedgehog, and Wnt signaling pathways as well as differential expression of the homeobox (HOX) transcription factors have been implicated in hematopoiesis (Argiropoulos and Humphries, 2007; Bhardwaj et al., 2001; Duncan et al., 2005; Milner and Bigas, 1999; Staal and Luis, 2010). Therefore, a highly complex signaling network of numerous intrinsic factors regulates hematopoiesis, and elucidation of these pathways continues to be a major topic of scientific investigation.

Cells within the HSC niche secrete extracellular molecules, such as cytokines and chemokines, which are also critical for hematopoiesis (Borish and Steinke, 2003). These extracellular signals function by stimulating their respective receptors and activating specific signaling cascades within immature hematopoietic cells. These signaling cascades may affect proliferation, differentiation, or apoptosis. For example, interleukin-3 (IL-3) and GM-CSF are cytokines that induce cell proliferation (de Groot et al., 1998), while Flt-3

ligand and Kit ligand (or SCF) protect cells from apoptosis (Murray et al., 1999). Likewise, at each stage of hematopoiesis, the cells can be characterized and identified by specific cell surface markers (Majeti et al., 2007; Passegue et al., 2003).

The sum of both internal and external cues determines whether a hematopoietic cell is quiescent, proliferates, differentiates, self-renews, or undergoes apoptosis. The balance of proliferation, differentiation, and apoptosis is critical to produce functional blood cells and to maintain hematopoiesis.

1.1.2 Hematological malignancies

As previously discussed, the balance of HSC self-renewal or differentiation along specific blood lineages is maintained via intrinsic and extrinsic regulatory pathways. Improper regulation of these cellular programs can alter the balance of normal hematopoiesis and ultimately lead to the development of hematological malignancy, such as leukemia. Leukemias encompass a heterogeneous group of hematological diseases arising from deregulated proliferation of hematopoietic cells that are unable to differentiate into functional blood cells (Lightfoot et al., 2008). Leukemic cells continue to self-renew and propagate themselves, resulting in displaced normal hematopoietic cells in the peripheral blood and bone marrow and infiltration into the spleen, lymph nodes, and other organs. Therefore, the generation of leukemia has severe consequences that affect normal processes, resulting in anemia, impaired immunity, and disrupted blood clotting (Lightfoot et al., 2008).

Leukemias are classified into different subtypes according to cellular immunophenotype and degree of maturation (Foon and Todd, 1986). Acute leukemias are characterized by immature blast-like cells, while the cells of chronic leukemias are more mature. Both acute and chronic leukemias can be divided into myeloid or lymphoid categories based on the lineage of the blast cells. Using French-American-British subtype nomenclature, acute leukemias are further classified by cellular morphology (Bennett et al., 1976).

Leukemias are thought to be clonal diseases propagated by leukemic stem cells (LSCs) (Huntly and Gilliland, 2005; Passegue et al., 2003). LSCs are defined as transformed hematopoietic cells that have the stem cell capacity for self-renewal but do not differentiate and have impaired functional properties (Passegue et al., 2003). Therefore, LSCs have indefinite proliferation potential and drive the formation and growth of the leukemia. Evidence for LSCs arose from studies showing that only a small subset of murine leukemic cells is capable of extensive proliferation in colony-forming assays *in vitro* and in transplantation assays *in vivo* (Bruce and Van Der Gaag, 1963; Park et al., 1971). Likewise, studies of human leukemias found that only a small fraction of cells (0.2-1%) is capable of propagating the disease in immunocompromised mice (Bonnet and Dick, 1997; Kamel-Reid et al., 1989; Lapidot et al., 1994; McCune et al., 1988). This clonogenic population was named a LSC because of its similarity to the normal hematopoietic lineage (Huntly and Gilliland, 2005). Because both cancers and stem cells

have the ability to undergo unlimited self-renewal, it has been proposed that leukemias are initiated by transforming events that take place in HSCs (Reya et al., 2001). Alternatively, leukemias may also arise from more committed progenitors that have reacquired enhanced self-renewal capabilities (Cozzio et al., 2003; Huntly et al., 2004; Passegue et al., 2003). Because the persistence of LSCs is responsible for disease maintenance and recurrence, identification and therapeutic targeting of LSCs is thought to be necessary for complete ablation of the disease.

Many developmental processes that are critical for normal hematopoiesis become perturbed during leukemogenesis. Leukemias are propagated by somatic mutations that give rise to a transformed clonal population of cells. To become fully malignant, cells must acquire mutations that support unlimited proliferation, prevention of differentiation, and suppression of apoptosis (Passegue et al., 2003). These transforming events often arise from misregulation of key regulatory pathways. For example, proper control of the *HOX* genes is important for both normal hematopoiesis and leukemogenesis.

1.1.3 Homeobox (*HOX*) genes

The clustered *HOX* family of homeobox genes is an evolutionarily conserved set of genes that encode DNA-binding transcription factors. In mammals, there are 39 *HOX* genes organized into four genomic clusters (A-D) located on four different chromosomes (**Figure 2A**). Each cluster is a paralog, and generally the paralogs have a high degree of

sequence similarity and functional redundancy (He et al., 2011). The arrangement of *HOX* genes into clusters allows for enhancer sharing, which enables a precise spatial and temporal coordination of expression during development (Alharbi et al., 2012).

HOX genes are integral regulators of temporospatial development along the anterior-posterior body axis of animal embryos (Krumlauf, 1994). There are three major modes of regulation that are necessary for ordered cluster expression during development (Shah and Sukumar, 2010). First, the position of a *HOX* gene 3′ to 5′ within a cluster corresponds to its expression along the anterior-posterior axis. Generally, 3′ genes are expressed in anterior tissues, and 5′ genes are expressed in posterior tissues. Second, *HOX* genes are normally expressed temporally in an order corresponding to their positions from 3′ to 5′ within each cluster. Therefore, 5′ *HOX* genes are generally expressed later in development. Finally, *HOX* genes positioned more 5′ in the cluster have dominant phenotypes to those more 3′. Loss of function mutations of 5′ *HOX* genes generally result in more severe abnormalities.

The homeobox genes direct embryonic development by regulating the expression of numerous target genes. All HOX proteins contain a highly conserved 60 aa helix-turn-helix DNA-binding domain, called the homeodomain (Gehring et al., 1994). Although the homeodomain binds DNA in a sequence-specific manner, it lacks substantial affinity and specificity. Therefore, HOX proteins interact with transcriptional co-factors to increase DNA-binding affinity and target specificity (Mann et al., 2009). The best studied co-

factors are members of the TALE (three amino acid loop extension) family of proteins, which in mammals include the PBX and MEIS families (Shen et al., 1996; Shen et al., 1997). Like HOX proteins, PBX and MEIS co-factors also have critical roles in development and hematopoiesis (Argiropoulos et al., 2007; Pillay et al., 2010; Pineault et al., 2002).

1.1.3.1 *HOXA* genes regulate self-renewal and differentiation of hematopoietic cells

In addition to their roles in embryogenesis, *HOX* genes of the A, B, and C clusters are also expressed in adult hematopoietic cells (Argiropoulos and Humphries, 2007). As in early development, hematopoietic expression of *HOX* genes is spatially and temporally regulated. Generally, 3′ *HOX* genes are highly transcribed in HSCs and immature progenitor cells, while 5′ genes are expressed during commitment. The *HOX* clusters are differentially expressed in each hematopoietic lineage, with *HOXC* in lymphoid cells, *HOXB* in erythroid cells, and *HOXA* in myeloid cells (Alharbi et al., 2012). For the purposes of this dissertation, we will primarily focus on the role of the *HOXA* cluster genes in normal and malignant hematopoiesis.

While genes of the *HOXA* cluster are highly transcribed in HSCs and immature progenitor cells, their expression is gradually down-regulated during differentiation and maturation (**Figure 2B**) (Pineault et al., 2002; Sauvageau et al., 1994). Numerous overexpression and knock-down studies have been performed to determine the effects of *HOXA* gene expression on hematopoiesis. Overexpression of *HOXA5* in human

hematopoietic progenitors results in an increased number of lineage-committed myeloid progenitors (Crooks et al., 1999; Fuller et al., 1999). Likewise, ectopic expression of *HOXA10* blocks differentiation of B- and T-cells and induces hyperproliferation of immature myeloid cells (Buske et al., 2001; Magnusson et al., 2007). Of all the *HOXA* genes, *Hoxa9* is most highly expressed in murine HSCs and progenitors, and its expression is down-regulated during differentiation (Pineault et al., 2002; Sauvageau et al., 1994). *Hoxa9*-deficient mice have defects along multiple hematopoietic lineages (Izon et al., 1998; Lawrence et al., 1997). Importantly, *Hoxa9*-deficient HSCs cannot repopulate lethally irradiated recipients after bone marrow transplantation (Lawrence et al., 2005). Conversely, overexpression of *Hoxa9* enhances HSC regeneration and self-renewal *in vivo* (Thorsteinsdottir et al., 2002). Similar results have been observed in *Hoxa5* and *Hoxa7* knockout mouse models (Fuller et al., 1999; So et al., 2004). While *Hoxa9* has the most profound effect, each of these knockout mice also has impaired HSC self-renewal and impaired differentiation.

The mechanism by which *HOX* genes regulate hematopoiesis is not fully understood. Genome-wide analyses have revealed that *HOX* genes can feedback and activate themselves or other *HOX* genes. For example, HOXA9 positively regulates transcription of *HOXA7* and *HOXA10* as well as its co-factors *PBX3* and *MEIS1* (Faber et al., 2009). HOXA9 was also found to positively regulate *Pim1*, *ID2*, *Flt3*, and *Cdk6*, genes involved in proliferation, survival, and oncogenesis (Gwin et al., 2010; Hu et al., 2007;

Huang et al., 2012; Nagel et al., 2010). Interestingly, HOXA9 also down-regulates genes involved in apoptosis and differentiation, such as *BIM* and *Runx1* (Huang et al., 2012; Nagel et al., 2010). In addition to HOXA9, HOXA10 has been shown to have similar downstream effects on the expression of pro-proliferation and anti-apoptotic genes (Alharbi et al., 2012; Bromleigh and Freedman, 2000; Magnusson et al., 2007).

The upstream regulation of *HOX* genes is critical for the orderly progression of hematopoiesis. Because *HOX* genes are expressed in clusters, changes in chromosome organization can have profound effects on their transcriptional regulation. Therefore, upstream regulators of *HOX* genes include factors that mediate this process, such as members of the Polycomb (PcG) and Trithorax groups (Beuchle et al., 2001; Ernst et al., 2004; Schuettengruber et al., 2007). PcG proteins encompass a large family of chromatin remodelers that generally function as transcriptional repressors. Knockout models of PcG proteins have impaired HSC function, which is thought result from dysregulated *HOX* gene transcription (Smith et al., 2011b; Takihara, 2008). Conversely, the mixed lineage leukemia (*MLL*) gene is a member of the Trithorax group of chromatin modifiers (Hanson et al., 1999). MLL is a histone H3 lysine 4 (H3K4) methyltransferase, which transcriptionally maintains active *HOX* gene expression during hematopoiesis (Milne et al., 2002). *Mll*-deficient mice have reduced *Hox* expression and dramatic reduction in HSCs and HPs (Ernst et al., 2004; Jude et al., 2007; McMahon et al., 2007). Strikingly, these hematopoietic deficiencies can be rescued by ectopic expression of *Hoxa9* or *Hoxa10*

(Ernst et al., 2004). Therefore, the balanced regulation of *HOX* expression by PcG and Trithorax proteins is critical for normal hematopoiesis.

1.1.3.2 Altered *HOXA* gene expression contributes to the pathogenesis of acute leukemia

Because *HOXA* genes are critical regulators of hematopoiesis, their perturbation can have profound effects on proliferation and differentiation of hematopoietic cells (**Figure 2B**). As discussed previously, an imbalance of proliferation, differentiation and apoptosis ultimately contributes to the development of leukemia. Therefore, altered expression of *HOX* genes may directly contribute to leukemogenesis. The role of *HOX* genes in hematological malignancies is supported by the frequent observation that they are overexpressed in human acute myeloid leukemias (AMLs). Overexpression of *HOX* genes is associated with unfavorable prognoses, and specifically, high levels of *HOXA9* are correlated with poor outcomes and a higher chance of relapse (Golub et al., 1999).

Overexpression of *HOXA* genes in leukemia occurs either through direct perturbation of the cluster or by deregulation of an upstream effector. An example of the former is the involvement of *HOX* genes in chromosomal translocations to generate chimeric fusions (discussed further in Section 1.2). Specifically, *NUP98*, a member of the nuclear pore family, is found in leukemogenic translocations with *HOXA9*, *HOXA11*, and *HOXA13* (Fujino et al., 2002; Nakamura et al., 1996; Suzuki et al., 2002). Expression of *NUP98-HOX* fusions in mice causes upregulation of other *HOXA* genes and leads to the generation of AML (Ghannam et al., 2004; Kroon et al., 2001; Pineault et al., 2005).

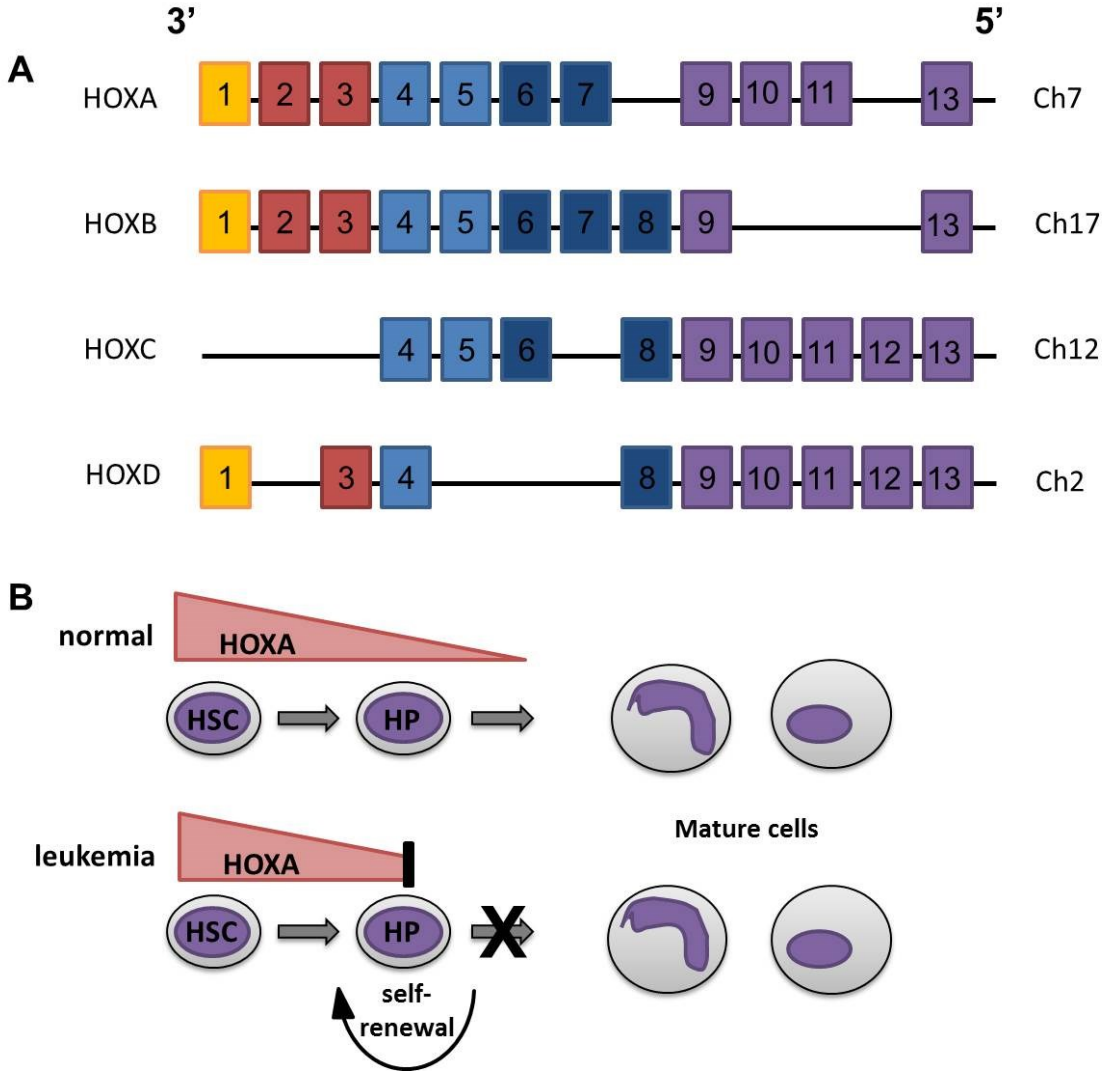

Figure 2: Organization of homeobox (*HOX*) cluster genes and the role of *HOXA* genes in normal and malignant hematopoiesis

(**A**) Schematic of the four human *HOX* clusters (A, B, C, and D) located on separate chromosomes (Ch). Across the clusters, humans have a total of 39 *HOX* genes. The genes can be aligned into paralogous groups (same colors) based on sequence homology. *HOX* genes are expressed spatially and temporally corresponding to their positions 3′ to 5′ within each cluster. (**B**) The *HOXA* cluster genes are important for hematopoiesis. Normally, *HOXA* genes are highly expressed in immature HSCs and progenitors, and their expression is turned off upon differentiation into mature blood cells. In leukemias, due to a various factors, a block in *HOXA* down-regulation results in increased self-renewal and inhibition of differentiation.

In addition, chromosomal perturbations involving the upstream *HOX* regulator, *MLL* are frequently found in human acute leukemias (Slany, 2009). Gene expression analyses of *MLL* leukemias have revealed elevated levels of *HOXA5-A11,* and the co-factor *MEIS1* (Armstrong et al., 2002). Importantly, *MLL* fusion-mediated transformation has been shown to be dependent on aberrant expression of the *Hoxa* genes (Ayton and Cleary, 2003; Okada et al., 2005).

In addition to the observation that *HOX* genes are overexpressed in acute leukemias, direct experimental evidence has verified the oncogenic potential of HOX proteins. First, retroviral expression of individual *HOXA* genes in murine bone marrow leads to increased clonogenic capacity and loss of differentiation of HPs *in vitro* (Bach et al., 2010). Likewise, transduction with *HOXA9* or *HOXA10* confers a growth advantage and leads to long latency leukemia, while co-overexpression of *MEIS1* collaborates to form a fully penetrant leukemia *in vivo* (Kroon et al., 1998; Thorsteinsdottir et al., 1997). Together, these studies suggest that overexpression of *HOXA* genes is sufficient for leukemogenesis. Altogether, the observations that *HOXA* genes are up-regulated in acute leukemias and can propagate the disease on their own suggest that misregulation of the *HOX* pathway is a common and driving mechanism of leukemic transformation.

1.2 Chromosomal translocations and hematologic malignancies

In 1914, Theodore Boveri theorized that acquired chromosome abnormalities in a single cell are causal factors in the formation of a tumor (Boveri, 2008). Almost a century later, it is widely agreed that genomic alterations can initiate and maintain cancers at the cellular level. There are many types of somatic mutations that are common in cancers, including point mutations, insertions, deletions, amplifications, copy number changes, and chromosome translocations. Cytogenetic analyses have revealed that epithelial tumors typically display a complex pattern of numerous chromosomal aberrations, whereas hematologic malignancies often contain few gross genomic abnormalities (Aplan, 2006). Importantly, these chromosome changes in leukemia usually reflect a single 'hit', which occurs early and is considered a driver of the disease (Greaves and Wiemels, 2003). The most well-studied driver mutation found in hematologic malignancies is the chromosomal translocation.

Nonrandom, somatically acquired chromosomal translocations are found in up to 65% of acute leukemias and lymphomas (Crans and Sakamoto, 2001). Chromosomal translocations are caused by re-joining DNA double strand breaks on heterologous chromosomes leading to chromosomal rearrangement. Oncogenic chromosome translocations involve recombination or juxtapositioning of normally separate genes. This can result in either altered expression of an oncogene or formation of a hybrid gene that encodes a fusion protein with altered function (Rabbitts, 1994). An example of the former

is the t(8;14) translocation associated with Burkitt's lymphoma, which juxtaposes the oncogene *CMYC* and the enhancer region of the immunoglobulin heavy chain gene locus, resulting in abnormally high levels of *CMYC* expression (Li et al., 2003; Taub et al., 1982). For the latter, the t(9;22) translocation fuses the *BCR* gene in frame with the *ABL1* gene, resulting in the *BCR-ABL1* oncoprotein, a hallmark of chronic myelogenous leukemia (Melo, 1996; Stam et al., 1985).

The study of leukemic chromosomal translocations has led to a number of important discoveries, both therapeutically and biologically. Chromosomal translocations are important factors for determining disease prognosis and selecting the most relevant treatments. Cloning of translocation breakpoints has led to the discovery of many critical genes involved in leukemogenesis, and resultant altered proteins form the basis for targeted therapeutics (Rowley, 2008). Characterization of these leukemic fusion genes provide insights into the mechanisms that lead to malignant transformation. For example, analysis of the *BCR-ABL1* fusion gene highlighted the role of tyrosine kinases in leukemic transformation and led to the development of the inhibitor imatinib (Druker et al., 2001). Therefore, it is of great biologic and therapeutic importance to study and characterize genes involved in chromosomal translocations.

1.2.1 Translocations involving the *CALM* and *AF10* genes

The t(10;11)(p13;q14) chromosomal translocation fuses the Clathrin Assembly Lymphoid-Myeloid leukemia gene (*CALM*, also known as *PICALM*) in-frame to *AF10*

(also known as *MLLT10*). The *CALM-AF10* fusion gene was first identified in the U937 cell line, which was derived from a patient with diffuse histiocytic lymphoma (Dreyling et al., 1996). Subsequently, *CALM-AF10* transcripts have been identified in patients with AML, T-cell acute lymphoblastic leukemia (T-ALL), undifferentiated acute leukemia, and malignant lymphoma (Bohlander et al., 2000; Dreyling et al., 1998). Although rare, malignancies with *CALM-AF10* fusion transcripts are generally immature and are associated with poor prognoses (Asnafi et al., 2003; Borel et al., 2012).

Using RNA interference in U937 cells, it was determined that *CALM-AF10* is necessary for cellular proliferation and xenotransplantation into mice (Okada et al., 2006). Retroviral transduction of *CALM-AF10* causes AML in a murine bone marrow transplantation model with a latency of 110 days (Deshpande et al., 2006). Similarly, *CALM-AF10* transgenic mice develop acute leukemia at a median age of 12 months (Caudell et al., 2007). Healthy *CALM-AF10* mice exhibit inhibition of myeloid and T-cell maturation, while the leukemias are mostly of myeloid origin (Caudell et al., 2007). Together, these results suggest that expression of *CALM-AF10* is necessary and sufficient for leukemogenesis. However, because mice display a long latency prior to the onset of leukemia, additional genetic events may be necessary for complete leukemic transformation (Caudell and Aplan, 2008).

Hematopoietic tissues from both clinically healthy and leukemic *CALM-AF10* mice display up-regulated *Hoxa5*, *Hoxa7*, *Hoxa9*, *Hoxa10* and *Meis1* expression (Caudell et

al., 2007). Importantly, Dik and colleagues reported that *HOXA5*, *HOXA9*, and *HOXA10* are up-regulated in the leukemic cells of patients with *CALM-AF10* fusions (Dik et al., 2005). *CALM-AF10*-mediated transformation is dependent on intact *Hox* expression, as the fusion gene cannot transform *Hoxa5*-deficient murine HPs *in vitro* (Okada et al., 2006). The elevated *HOX* expression patterns are similar to those observed in *MLL* translocations, suggesting a common oncogenic pathway.

Chromosomal rearrangements involving the *MLL* gene have been associated with a heterogeneous group of myeloid, lymphoid, and mixed lineage leukemias (Harper and Aplan, 2008). *MLL* rearrangements are found in up to 10% of adult and 80% of infant leukemias (Bernt and Armstrong, 2011; Harper and Aplan, 2008). Like *CALM-AF10*, *MLL* leukemias are characterized by up-regulated *HOXA* cluster expression (Armstrong et al., 2002). *MLL* is regarded as a 'promiscuous' oncogenic fusion gene because over 70 different translocation partners have been identified. Intriguingly, both *CALM* and *AF10* have been identified as *MLL* fusion partners in patients with acute leukemias (Chaplin et al., 1995b; Wechsler et al., 2003). The observation that *CALM* and *AF10* are involved in separate leukemic fusions with each other and with *MLL* suggests that disruption of their normal functions contributes to leukemogenesis.

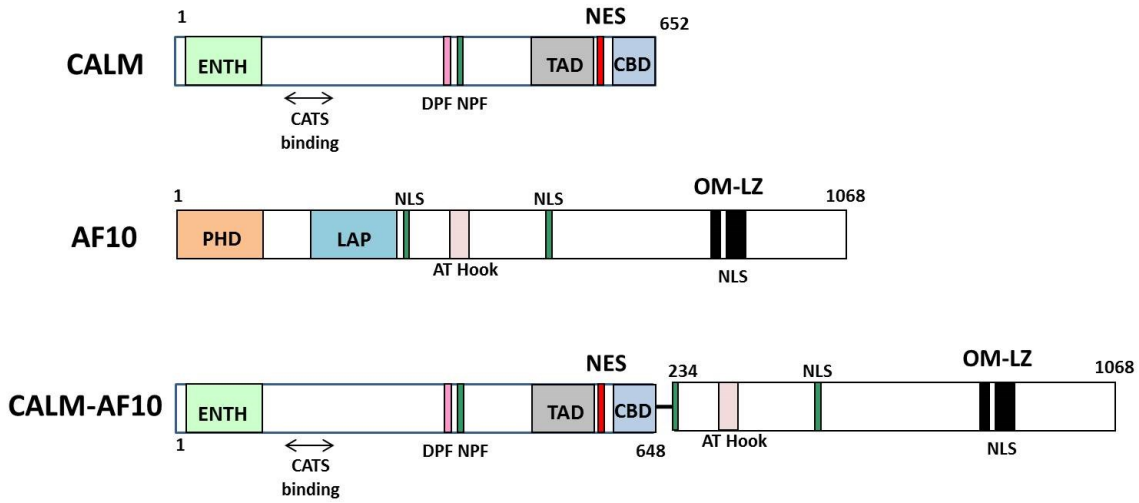

Figure 3: CALM, AF10, and CALM-AF10 proteins

Schematic representation of CALM, AF10, and CALM-AF10 fusion proteins and the various domains are shown. CALM interacts with the plasma membrane via the ENTH domain, and with various endocytic proteins via the DPF, NPF, and Clathrin Binding Domain (CBD). The region containing a putative transcriptional activation domain (TAD) is in grey. Here, we find that CALM contains a functional nuclear export signal (NES; red) that is critical for leukemogenesis. AF10 has plant homeodomain (PHD) and leukemia-associated protein (LAP) domains that are lost in the CALM-AF10 fusion. AF10 can interact with DNA via the AT hook and contains numerous nuclear localization sequences (NLS) that contribute to its nuclear localization. The octapeptide motif-leucine zipper (OM-LZ; black bars) region is both necessary and sufficient for leukemogenesis. CALM-AF10 fusions consistently include most of the CALM coding region (including the NES) and the carboxy-terminus of AF10 (including OM-LZ).

1.3 AF10

The *AF10* (officially denominated *MLLT10*) gene was first identified as a fusion partner for the *MLL* gene in patients with the recurrent t(10;11)(p13;q23) translocation (Chaplin et al., 1995b). AF10 encodes a 1068 aa nuclear protein that is a member of a small but highly conserved family that includes AF17, BR140 and CEZF (*C. elegans*) (Chaplin et al., 1995a; Linder et al., 2000). AF10 is thought to function as a transcription factor and

contains conserved plant homeodomain (PHD) fingers in the N-terminal (**Figure 3**) (Linder et al., 1998; Saha et al., 1995). PHD fingers are present in chromatin modifiers, such as CBP, MLL, TRX, and Polycomb group proteins, suggesting a role for AF10 in chromatin-mediated gene expression (Aasland et al., 1995). AF10 also contains a leukemia-associated protein (LAP) domain that functions in homo-oligomerization, an AT-hook motif that mediates binding to DNA, and several nuclear localization signals (NLS) that direct AF10 localization to nuclei (Aravind and Landsman, 1998; Linder et al., 1998; Linder et al., 2000). The carboxy-terminus of AF10 contains an octapeptide motif (consensus sequence EQLLERQW) followed by a leucine zipper (OM-LZ) protein-protein interaction domain (**Figure 3**) (Chaplin et al., 1995a; DiMartino et al., 2002; Okada et al., 2005). As discussed further in this dissertation, the OM-LZ domain has been shown to mediate interactions between AF10 and GAS41, IKAROS, and DOT1L.

CALM-AF10 and MLL-AF10 translocations lose the PHD and LAP domains of AF10 but consistently include the carboxy-terminal OM-LZ domain (**Figure 3**) (Dreyling et al., 1996; Narita et al., 1999; Silliman et al., 1998). The OM-LZ domain has been shown to be necessary and sufficient for both *CALM-AF10*- and *MLL-AF10*-mediated leukemogenesis (Deshpande et al., 2011; DiMartino et al., 2002; Okada et al., 2005; Okada et al., 2006). Mutation of this domain abrogates the ability to transform murine HPs *in vitro*, and retroviral expression of artificial CALM-OM-LZ or MLL-OM-LZ fusions are sufficient for leukemic transformation. Therefore, the OM-LZ domain is the critical

structural contribution of AF10 to leukemogenesis; however, the precise mechanisms by which it mediates oncogenic immortalization are not fully understood.

1.3.1 AF10 OM-LZ binding partners

Structural analysis revealed that the OM and LZ domains form alpha-helices, which are separated by a non-helical peptide segment (DiMartino et al., 2002). This region of AF10 has been shown to function as a protein interaction domain. Therefore, the finding that the OM-LZ domain is necessary and sufficient for leukemogenesis suggests that AF10 imparts its transformation potential through interaction with another protein(s). Known OM-LZ binding partners include GAS41, IKAROS, and DOT1L, and each of these proteins is involved in gene regulation (Debernardi et al., 2002; Greif et al., 2008; Okada et al., 2005). DOT1L has been the most extensively studied AF10 binding partner in leukemogenesis, but little is known about roles of GAS41 or IKAROS. Below, each of the known OM-LZ binding partners will be discussed separately.

1.3.1.1 GAS41

Using yeast 2 hybrid screening techniques, the leucine zipper motif of AF10 was found to interact with the glioma-amplified sequence 41 (GAS41) protein (Debernardi et al., 2002). GAS41 is the mammalian homolog of the *S. cerevisiae* ANC1 protein. ANC1 is an integral member of the TFIID and TFIIF basal transcription factor complexes, as well as a component of the SWI/SNF chromatin remodeling complex (Cairns et al., 1996; Kabani et al., 2005). Like ANC1, mammalian GAS41 has been shown to bind the TFIIF

complex as well as INI, a component of the SWI/SNF complex (Debernardi et al., 2002; Heisel et al., 2010). Interestingly, the N-terminal region of GAS41 is homologous to the mammalian proteins AF9 and ENL (Debernardi et al., 2002). In addition to being well-characterized MLL leukemic fusion partners, AF9 and ENL are components of the transcriptional elongation complex (Lin et al., 2010). Altogether, these findings suggest that GAS41 may have a global role in chromatin structure and gene transcription.

Little is known about the role of the GAS41-AF10 interaction in AF10 fusion protein-mediated leukemias. It is tempting to speculate that AF10 may aberrantly recruit transcriptional or epigenetic complexes via its interaction with GAS41. However, additional research is necessary to elucidate the potential contribution of GAS41 to *CALM-AF10* leukemogenesis.

1.3.1.2 IKAROS

A separate yeast 2 hybrid study determined that the AF10 carboxy-terminus binds the lymphoid regulator, IKAROS (Greif et al., 2008). IKAROS interacts with chromatin remodeling factors and is involved in transcriptional regulation and cell cycle control (Gomez-del Arco et al., 2004; Kim et al., 1999). IKAROS normally localizes to the nucleus and has been shown to bind pericentromeric heterochromatin (Cobb et al., 2000). Its role in transcriptional regulation is necessary for B- and T-cell development (Georgopoulos et al., 1994; Winandy et al., 1995). Likewise, the *Ikaros* gene is frequently deleted in ALL (Georgopoulos et al., 1992).

Using *in vitro* and *in vivo* assays, AF10 was found to interact with IKAROS via the OM-LZ domain. While IKAROS and AF10 co-localize in the nucleus, IKAROS becomes primarily cytoplasmic when co-expressed with CALM-AF10 (Greif et al., 2008). Therefore, CALM-AF10 may alter the subcellular localization of IKAROS from the nucleus to the cytoplasm. Mislocalization of IKAROS could have profound effects on its transcriptional targets. Indeed, while IKAROS normally represses transcription in GAL4 assays, co-expression of CALM-AF10 reduced IKAROS-mediated repression by 11-fold (Greif et al., 2008). Although the importance of the AF10-IKAROS interaction in CALM-AF10 leukemias remains unclear**,** loss of IKAROS function via cytoplasmic localization may lead to disturbed lymphoid differentiation, which could contribute to transformation (Greif and Bohlander, 2011).

1.3.1.3 DOT1L

Finally, the AF10 OM-LZ domain has been shown to interact with DOT1L (disruptor of telomeric silencing 1-like) (Okada et al., 2005). DOT1L is the sole mammalian histone H3 lysine 79 (H3K79)-specific methyltransferase (Jones et al., 2008). Unlike most histone methyltransferases, DOT1L does not contain a SET domain, and instead, the catalytic domain is an S-Adenosyl-L-methionine (SAM) motif (Min et al., 2003). H3K79 methylation is a chromatin modification ubiquitously associated with actively transcribed genes and can be either mono-, di-, or tri-methylated (Frederiks et al., 2008; Steger et al., 2008). To date, a specific H3K79 demethylase has not been identified

(Nguyen and Zhang, 2011). Therefore, H3K79 regulation is considered to be entirely dependent on DOT1L and histone turnover (Daigle et al., 2011).

Because DOT1L interacts with the critical AF10 OM-LZ domain, it has been suggested that AF10 serves as a bridge to recruit DOT1L to CALM-AF10 fusions (Nguyen and Zhang, 2011). To examine the necessity of DOT1L for *CALM-AF10*-mediated transformation, Okada et al. overexpressed either wild-type or H3K79 methyltransferase-defective DOT1L in *CALM-AF10* transformed murine bone marrow cells. While wild-type DOT1L slightly increased colony formation, mutant DOT1L suppressed the colony-forming potential of CALM-AF10 (Okada et al., 2006). In addition, they found that H3K79 di-methylation is enriched at the promoter regions of the *HOXA* cluster genes in *CALM-AF10* leukemic cells (Okada et al., 2006). Likewise, CALM-AF10 itself was shown to bind within the coding region of *Hoxa5* (Okada et al., 2006). The recruitment of CALM-AF10 and DOT1L to the *Hoxa* locus corresponds with increased transcription of these cluster genes, which are critical mediators of leukemogenesis.

In addition to aberrant H3K79 methylation on the *Hoxa* locus, *CALM-AF10*-expressing cells display a global reduction of di-me H3K79 across the genome (Lin et al., 2009). CALM-AF10-induced H3K79 hypomethylation is dependent on the OM-LZ domain, suggesting that AF10 dissociates DOT1L from chromatin (Lin et al., 2009). Although the mechanism by which this occurs remains unknown, H3K79

hypomethylation has been correlated with increased chromosomal instability, which may contribute to leukemogenesis.

Altogether, these data suggest that the AF10-DOT1L interaction contributes to leukemogenesis. However, the precise mechanisms by which abnormal H3K79 methylation patterns are established and how these epigenetic changes contribute to AF10-rearranged leukemias remain to be elucidated. Likewise, insights into the endogenous functions for the AF10-DOT1L interaction may contribute to our understanding of how these proteins are perturbed in leukemia.

1.3.2 Physiological functions of AF10

1.3.2.1 Transcriptional elongation and epigenetic modification

As discussed, AF10 has been shown to interact with many proteins that are involved in transcriptional and epigenetic regulation. These findings suggest that AF10 may have a broad role in gene regulatory processes. Indeed, several groups have documented a potential involvement of AF10 in transcriptional elongation. First, Bitoun et al. characterized a complex that contains DOT1L, AF4, ENL, AF9, AF10, and P-TEFb (Bitoun et al., 2007). P-TEFb phosphorylates the RNA Pol II carboxy-terminus, which is required for the transition from transcriptional initiation to elongation (Marshall et al., 1996). Overexpression of each component, including AF10, increased H3K79 methylation and P-TEFb-dependent transcription elongation (Bitoun et al., 2007). These results

suggest a link between H3K79 methylation and transcriptional elongation; however, the mechanistic connection between methylation and elongation remains unknown.

Another AF10 complex that contains DOT1L was also recently identified and named DotCom (Mohan et al., 2010). DotCom does not contain P-TEFb but rather components of the Wnt/β-catenin pathway. DotCom has also been shown to methylate H3K79 and increase transcriptional activation (Mohan et al., 2010). The DotCom components, DOT1L, AF10, and β-catenin were found to form a complex and activate downstream Wnt target genes during development in *Drosophila* (Mohan et al., 2010). Interestingly, a separate report characterized an AF10 complex with Tcf4/β-catenin and DOT1L in mouse small intestinal crypts, where it participates in the maintenance of intestinal cell homeostasis through the regulation of downstream Wnt target genes (Mahmoudi et al., 2010). Both studies found that siRNA-mediated knockdown of DOT1L or AF10 results in a global loss of H3K79 methylation, and it was proposed that AF10 directly tethers DOT1L to target genes (Mahmoudi et al., 2010; Mohan et al., 2010). Altogether, these studies show that endogenous AF10 is a critical mediator of DOT1L-mediated H3K79 methylation and transcriptional elongation.

1.3.2.2 Normal hematopoiesis

A recent study examined the expression pattern of AF10 during hematopoietic differentiation (Chamorro-Garcia et al., 2012). The authors found that while primary immature cells have high AF10 expression levels, AF10 is decreased in differentiated

macrophages. Both overexpression and knockdown of AF10 resulted in apoptotic cell death, suggesting that AF10 levels must be tightly controlled during hematopoiesis (Chamorro-Garcia et al., 2012). Similar results have been observed in *Dot1L*-deficient mice, corroborating the relationship between DOT1L and AF10 (Feng et al., 2010; Jones et al., 2008). Although further studies are necessary, these findings suggest that AF10 is important in early stages of hematopoiesis and may provide insight into how AF10-fusions cause hematological malignancies.

1.4 CALM

This section appears in modified form in: Conway A.E and Wechsler D.S. "PICALM" in Schwab M. (Ed.) Encyclopedia of Cancer: SpringerReference (2009).

The Clathrin Assembly Lymphoid Myeloid leukemia (*CALM*; also known as *PICALM*) gene encodes a ubiquitously expressed 652 aa protein with homology to AP180, a neuronal protein involved in clathrin assembly and endocytic vesicle formation (Dreyling et al., 1996; Prasad and Lippoldt, 1988). Like AP180, CALM contains many conserved domains including an epsin N-terminal homology (ENTH) domain, a DPF (Asp-Pro-Phe) motif, NPF (Asn-Pro-Phe) motifs and clathrin-binding sequences (**Figure 3**) (Scotland et al., 2012; Tebar et al., 1999). The ENTH domain allows for binding to phosphatidylinositol-4,5-bisphosphates, components of plasma membranes (Ford et al.,

2001). The DPF motif interacts with the clathrin adaptor protein AP2 (Kalthoff et al., 2002; Owen et al., 1999), and NPF motifs bind Eps15 homology domains found in several components of the endocytic machinery (Paoluzi et al., 1998). Likewise, CALM also binds clathrin heavy chain, and our laboratory has determined that this interaction is mediated by the extreme CALM carboxy-terminus (aa 583-652; **Figure 3**) (Scotland et al., 2012). Altogether, these structural domains suggest a role for CALM in clathrin-mediated endocytosis.

Although various breakpoints have been identified, the extreme carboxy-terminus of *CALM* is consistently joined with *AF10* in human leukemias (Narita et al., 1999; Silliman et al., 1998). Therefore, almost the entire CALM coding sequence is found in CALM-AF10 translocations, which has made it difficult to delineate the key functional domain of CALM for leukemogenesis (**Figure 3**). It was recently determined that a region encompassing the last 248 aa of CALM (aa 400-648) is sufficient for transformation when fused to full-length AF10 or the OM-LZ domain (Deshpande et al., 2011). While these findings are novel and begin to delineate the structural contribution of CALM, the specific domain of CALM necessary for leukemogenesis and the mechanism by which CALM imparts transformation potential to AF10 remain unknown.

1.4.1 Physiological functions of CALM

1.4.1.1 Clathrin-mediated endocytosis

Endocytosis is characterized by the internalization of plasma membrane-associated cargo molecules and subsequent trafficking through intracellular vesicle compartments or endosomes (Doherty and McMahon, 2009). The endosomes either recycle the cargo back to the cell surface or to lysosomes for degradation (Grant and Donaldson, 2009). One such trafficking mechanism is clathrin-mediated endocytosis (CME), in which adaptor proteins specifically recognize cargo to be internalized and initiate packaging into clathrin-coated vesicles (CCVs) (Schmid, 1997). Formation of CCVs and subsequent trafficking through the endosomes requires the association of many adaptor and accessory proteins (Owen et al., 2004). These proteins generally function as scaffolds between the plasma membrane, cargo molecules, and clathrin (Maldonado-Baez and Wendland, 2006).

CALM and its neuronal homolog, AP180, function as accessory proteins in CME (Hao et al., 1999; Tebar et al., 1999). Both CALM and AP180 contain ENTH domains that bind membrane inositol polyphosphates (Ford et al., 2001). CALM also contains NPF and DPF domains, which allow for binding to other endocytic adaptor proteins, such as AP2 (**Figure 3**) (Kalthoff et al., 2002; Paoluzi et al., 1998). Importantly, CALM and AP180 interact with clathrin and facilitate the formation of CCVs (Meyerholz et al., 2005; Morgan et al., 1999; Zhang et al., 1998). The size and shape of CCVs is maintained through a critical interaction between CALM, AP2, and clathrin (Meyerholz et al., 2005).

Because CALM can bind both endocytic adaptor proteins and membrane inositol polyphosphates, it is theorized that CALM is a scaffolding protein that mediates clathrin cage assembly. In support of this role, both overexpression and knock-down studies of CALM show inhibition of CME of transferrin receptor (TfR) or epidermal growth factor receptor (EGFR) (Meyerholz et al., 2005; Scotland et al., 2012; Tebar et al., 1999). Although the specific function of CALM in CME has not yet been determined, it is clearly important for the orderly progression of endocytosis at the plasma membrane.

In addition to the plasma membrane, CCVs also bud from the Golgi apparatus (Schmid, 1997). Formation of CCVs at the trans-Golgi network (TGN) requires clathrin and the AP1 complex (Traub et al., 1995; Wang et al., 2003). CALM co-localizes with clathrin at the plasma membrane at in the TGN, and overexpression of CALM results in a loss of clathrin accumulation in the TGN (Tebar et al., 1999). Therefore, in addition to its role in CME, stoichiometric levels of CALM are necessary for clathrin-mediated trafficking at the TGN.

1.4.1.2 Normal hematopoiesis and iron homeostasis

CALM has been shown to be involved in normal hematopoiesis and iron metabolism in the mouse (Klebig et al., 2003). *Calm*-deficient mice were generated by an N-ethyl-N-nitrosourea (ENU) saturation mutagenesis screen for mutations affecting developmental pathways (Potter et al., 1995). Mutagenesis of a defined region of mouse chromosome 7 led to growth retarded and "less fit" mice, and this allele was named

fitness1 (*fit1*). It was later determined that the ENU-induced mutations disrupted splice-donor sites within *Calm* resulting in exon deletions and premature termination codons, which truncated the Calm protein at the N-terminal within the ENTH domain (Klebig et al., 2003). These *fit1 (Calm*-deficient*)* mice not only exhibit *in utero* growth retardation, but they also suffer from defective hematopoiesis (Potter et al., 1997). Specifically, *fit1* mice have splenic hyperplasia, reduced erythroid and myeloid precursors, and reduced B-cell number. These mice also suffer from severe hypochromic anemia and display reduced serum iron levels and abnormal tissue iron distribution. In addition to *fit1*, a second *Calm*-deficient mouse model has been recently developed, and exhibits similar phenotypes of growth retardation, anemia, and defects in hematopoiesis (Suzuki et al., 2012). Because *Calm* is the only abnormality identified in these mice, it is clear that reduced levels of Calm protein contribute to defective hematopoiesis, iron metabolism, and growth *in vivo*.

The anemic state of the *Calm*-deficient mice suggests that CALM plays an important role in iron internalization and homeostasis. *In vitro* studies using mouse embryonic fibroblasts and hematopoietic cells derived from *Calm*-deficient mice have demonstrated that the rate of transferrin receptor endocytosis is perturbed in these cells (Scotland et al., 2012; Suzuki et al., 2012). Likewise, *Calm*-deficient cells have reduced intracellular iron levels and as a result, proliferate more slowly than their wild type counterparts (Scotland et al., 2012).

1.4.1.3 CALM undergoes nucleocytoplasmic shuttling

Although CALM is predominantly a cytoplasmic protein, it can undergo nucleocytoplasmic shuttling (Vecchi et al., 2001). About 50% of endogenous CALM protein becomes nuclear following treatment with Leptomycin B (LMB), which specifically inhibits nuclear export through the nuclear export receptor CRM1 (Kudo et al., 1998; Vecchi et al., 2001). As discussed further in this dissertation, CRM1 recognizes conserved nuclear export signal (NES) motifs on cargo proteins and mediates nuclear export in a Ran-GTP-dependent manner (Askjaer et al., 1998; Fornerod et al., 1997). Vecchi et al. determined that CALM binds CRM1 (with Ran-GTP) *in vitro*. Although CALM does not contain a classical nuclear localization signal (NLS), these results suggest that CALM must have a conserved CRM1-dependent NES (Okada et al., 2006).

CALM exhibits low level transcriptional activation in GAL4-based transcription assays (Vecchi et al., 2001). Structure-function studies have elucidated a putative transcriptional activation domain (TAD) in the carboxy-terminal region of CALM (Archangelo et al., 2006). Additionally, CALM associates with a novel nuclear protein termed CATS (CALM interacting protein expressed in Thymus and Spleen) (Archangelo et al., 2006). The function of CATS is not known, however its expression is cell-cycle dependent and induces transcription in GAL4 assays (Archangelo et al., 2008). In addition, CALM was found to interact with FHL2 (Four and a Half LIM domain protein 2), another nuclear protein involved in transcriptional regulation (Pasalic et al., 2011).

These observations suggest that in addition to its role in endocytosis, CALM shuttles to the nucleus and may have a transcriptional regulatory function. Intriguingly, a growing number of endocytic proteins have been shown to translocate to the nucleus and affect transcriptional processes (Borlido et al., 2009; Pyrzynska et al., 2009). At this time, it is unclear whether these are separate "moonlighting" roles or if the endocytic pathway directly signals to the nucleus (Pilecka et al., 2007).

1.5 Nuclear export pathway

While small molecules (less than 40 kDa) can passively diffuse across the nuclear envelope, larger molecules are transported by signal- and energy-dependent mechanisms (Gorlich and Mattaj, 1996). Active transport between the nucleus and cytoplasm occurs at the multi-protein nuclear pore complex (NPC) (**Figures 4-5**). The NPC is an eightfold symmetrical complex composed of approximately 30 different nucleoporins (Nups) and has a total molecular mass of 125 MDa (Akey, 1989; Cronshaw et al., 2002; Reichelt et al., 1990). The nuclear envelope is perforated by numerous NPCs, which allows for controlled bidirectional transport of hundreds of macromolecules (Fahrenkrog et al., 2004). Therefore, NPCs are considered to be gatekeepers of the nucleus (Capelson et al., 2010a; Wente, 2000).

Nucleocytoplasmic transport is a complex process that is carried out by a large family of transport receptors. These receptors are collectively called karyopherins and are

broken down into importins or exportins, depending on the direction of cargo transport (Terry et al., 2007). Following binding in the originating compartment, the transport complexes undergo stepwise translocation through the NPC to the destination compartment (**Figure 4**). Here, the cargo dissociates from the receptor, and the receptor is returned to the original compartment.

Importins and exportins recognize protein cargoes by specific signals called nuclear localization sequences (NLS) or nuclear export signals (NES), respectively (Kosugi et al., 2008; Lange et al., 2007; Lee et al., 2006; Ossareh-Nazari et al., 1997; Richards et al., 1996). Receptors interact with cargo either directly or indirectly through adaptor molecules, such as the small GTPase Ran. Ran plays a key role in determining the directionality of nuclear transport (Izaurralde et al., 1997; Nachury and Weis, 1999). The Ran GTPase-activating protein (GAP) is sequestered in the cytoplasm (Floer and Blobel, 1996; Gorlich et al., 1996), and the Ran guanine nucleotide exchange factor (GEF; called RCC1) is restricted to the nucleus (Klebe et al., 1995; Ohtsubo et al., 1989). Therefore, most nuclear Ran is in the GTP-bound form and cytoplasmic Ran is in the GDP-bound form (**Figure 4**). Importins release their cargo into the nucleus upon binding RanGTP; conversely, nuclear RanGTP promotes association of exportins with NES-containing cargo. This balance of Ran and its effectors is critical for proper loading and unloading of nuclear transport receptors (Tetenbaum-Novatt and Rout, 2010).

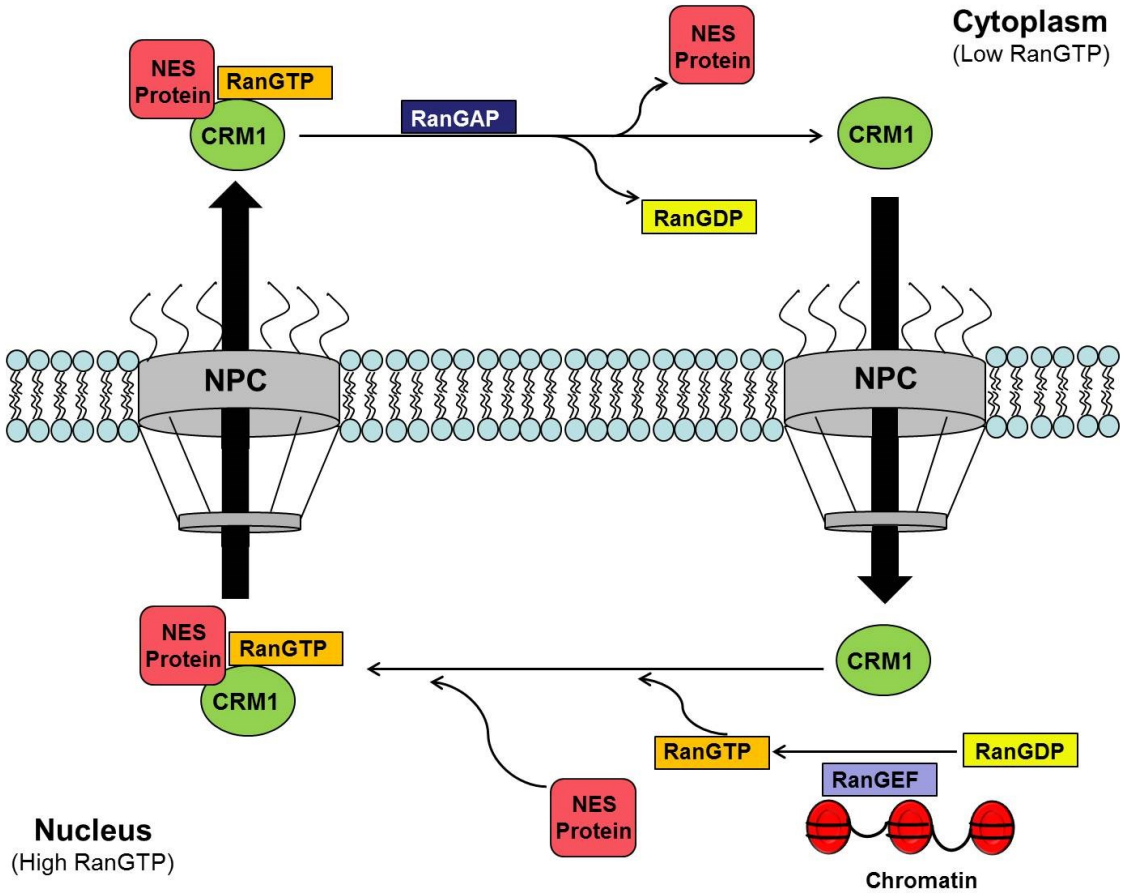

Figure 4: The CRM1-mediated nuclear export pathway

Nuclear export of a cargo protein (pink square) occurs by association of its nuclear export signal (NES) with the nuclear export receptor CRM1 (green circle). CRM1 efficiently binds its cargo along with Ran-GTP (orange rectangle) in the nucleus and shuttles through the nuclear pore complex (NPC). Ran-GDP (yellow rectangle) is phosphorylated by the Ran-GEF (RCC1; purple rectangle), which is bound to chromatin (red circles) to make Ran-GTP. Therefore, high levels of Ran-GTP are found in the nucleus and assist CRM1-mediated nuclear export. In the cytoplasm, RanGTP is dephosphorylated by the Ran-GAP (blue rectangle), leading to an accumulation of Ran-GDP in the cytoplasm. Ran-GDP is critical for cargo loading and efficient nuclear import (not shown). The balance of Ran is essential for loading and unloading of nuclear transport receptors.

1.5.1 CRM1 nuclear export receptor

CRM1 (Chromosomal maintenance 1; also known as Exportin1) facilitates the transport of various large macromolecules across the nuclear membrane to the cytoplasm, making it the major mammalian nuclear export receptor (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997). Specifically, CRM1 mediates nuclear export of proteins that contain a short, leucine-rich NES (**Figure 4**), which was first identified in HIV Rev and protein kinase A inhibitor (PKIA) (Fischer et al., 1995; Wen et al., 1995). Mouse and human CRM1 crystal structures reveal that CRM1 bends into a distorted toroid structure through which it interacts with RanGTP and NES-containing cargo molecules (Dong et al., 2009; Monecke et al., 2009). The affinities of cargoes for CRM1 vary drastically depending on the spacing of the hydrophobic residues within the NES (Guttler et al., 2010). Surprisingly, most substrates have a low binding affinity for CRM1-RanGTP (Kutay and Guttinger, 2005). Complexes between high-affinity NES motifs and CRM1 were found to accumulate at the NPC, suggesting that lower affinity for CRM1 is essential for efficient release into the cytoplasm (Engelsma et al., 2004). Furthermore, weak affinity interactions may prevent cargo from binding CRM1 in the absence of RanGTP and thus function as a means to regulate specificity (Kutay and Guttinger, 2005).

Leptomycin B (LMB) covalently binds to and alters a cysteine residue within the hydrophobic cleft of CRM1 (Kudo et al., 1999; Kudo et al., 1998; Nishi et al., 1994). LMB-

modified CRM1 cannot bind to NES-containing proteins that rely on this structure (Dong et al., 2009; Monecke et al., 2009). Therefore, LMB is a potent inhibitor of CRM1 and prevents CRM1-mediated nuclear export of many proteins (Nguyen et al., 2012). In mammalian cells, extended treatment with LMB causes a cell cycle arrest at both G1 and G2 phases (Yoshida et al., 1990).

1.5.2 Nucleoporins

The Nucleoporins (Nups) are the major components of the NPC and have precise roles in NPC assembly and function. Structural (scaffold) Nups contribute to the overall NPC architecture, and transmembrane Nups anchor the NPC to the nuclear envelope (**Figure 5**) (Alber et al., 2007; Hetzer et al., 2005). Interestingly, structural studies have shown that scaffold Nups are arranged in a similar fashion as clathrin coated vesicles, suggesting that share a common molecular architecture (Alber et al., 2007; Devos et al., 2004). A third class of Nups contain multiple phenylalanine glycine (FG), GLFG, or FxFG repeat motifs (collectively called FG-Nups) (Bayliss et al., 2000; Radu et al., 1995; Terry and Wente, 2009). FG-Nups are essential for controlling active translocation through the NPC by interacting with transport receptors (Isgro and Schulten, 2007a; Isgro and Schulten, 2007b). Specifically, CRM1-mediated translocation through the NPC is energy-independent and occurs by multiple low-affinity interactions with FG-Nups (Terry et al., 2007; Terry and Wente, 2009). FG-Nups are thought to function as a selective permeability barrier between the nucleus and cytoplasm (**Figure 5**) (Frey et al., 2006;

Ribbeck and Gorlich, 2001; Weis, 2007). In addition to their roles in mediating transport, many FG-Nups are mobile and shuttle on and off the NPC (Griffis et al., 2004; Rabut et al., 2004).

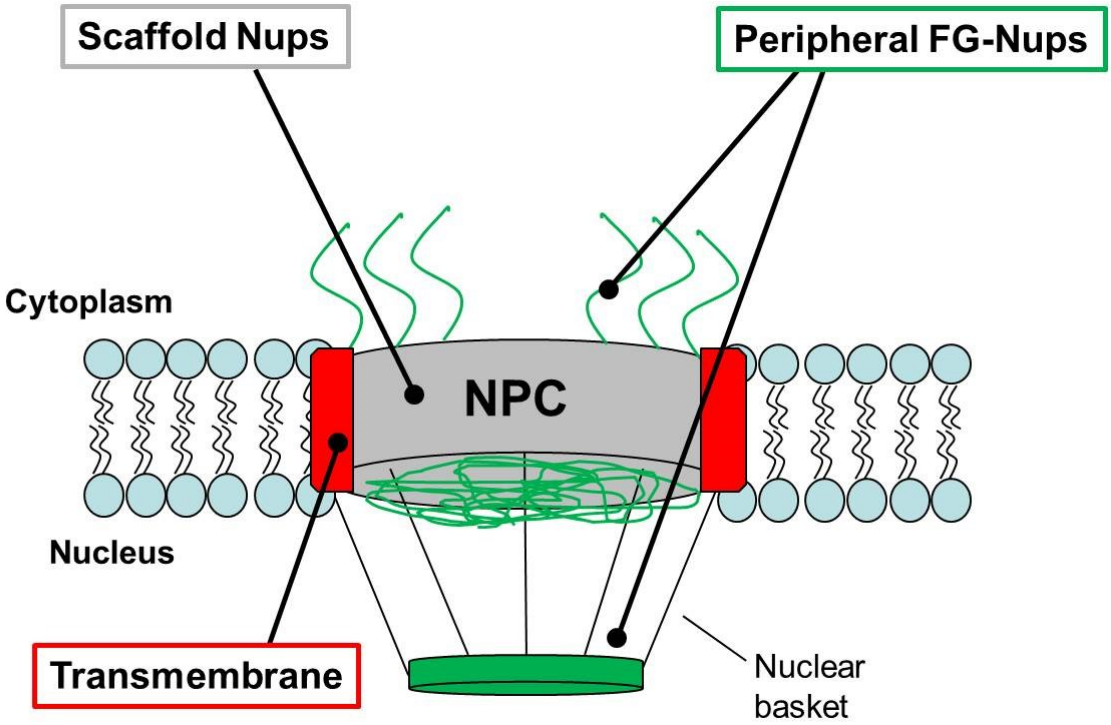

Figure 5: The Nuclear Pore Complex (NPC) is composed of nucleoporins (Nups)

The NPC is schematically illustrated to show its association with the nuclear envelope and localization of Nups. The NPC is composed of 30 different Nups, which can be classified into three different classes. The transmembrane Nups (red) function to physically tether the NPC to the nuclear membrane. The scaffold Nups (grey) contribute to the overall architecture of the NPC. Peripheral Nups can be found on either or both the nuclear and cytoplasmic face of the NPC. Most FG-Nups are peripheral Nups are found at the nuclear basket or cytoplasmic filaments to form a selective permeability barrier (depicted in green). The FG-Nups interact with nuclear transport receptors to help mediate nuclear import and export.

Some peripheral Nups have been shown to shuttle between the nuclear periphery and the nucleoplasm (Rabut et al., 2004). Dynamic movement of Nup98 and Nup153 off the NPC is dependent on active transcription (Griffis et al., 2004). It has been suggested that these dynamic Nups either help to deliver cargo to the NPC for active transport, or they may have a transport independent role in gene regulation (D'Angelo and Hetzer, 2008). In support of the latter, *Drosophila* Nup98 and Nup153 directly interact with active genes in the nuclear interior (Capelson et al., 2010b; Kalverda et al., 2010). Recently, human NUP98 was found to dynamically associate with the genome as well as promote epigenetic memory (Liang et al., 2013; Light et al., 2013). Although it is well established that the NPC can be major site of epigenetic and transcriptional regulation, these results suggest that some Nups may directly control gene expression within the nucleoplasm (Arib and Akhtar, 2011; Brown and Silver, 2007; Kohler and Hurt, 2010).

1.5.3 The nuclear export pathway and leukemogenesis

Components of the nuclear export pathway have been directly implicated in the pathogenesis of leukemia. Specifically, the *NUP98* and *NUP214* genes are sites of recurrent chromosomal translocations that result in oncogenic fusions (Gough et al., 2011; Lee et al., 2012). As discussed previously, *NUP98* fusions with *HOXA* genes result in AML through perturbation of downstream transcriptional pathways involved in proliferation and differentiation (Ghannam et al., 2004; Takeda et al., 2006). In addition to *HOXA* genes, NUP98 has also been found in translocations with numerous PHD-finger

proteins in human leukemias (Wang et al., 2009). Mechanistically, Nup98 was found to bind the histone acetyltransferase CBP/p300, resulting in aberrant acetylation and activation of *HOXA* genes (Kasper et al., 1999). Another nucleoporin involved in oncogenic translocations is NUP214. *NUP214-ABL1* fusions are present in ALLs, and localization to the NPC and interaction with other Nups are required for NUP214-ABL1-mediated transformation (De Keersmaecker et al., 2008). Intriguingly, a separate NUP214 fusion, SET-NUP214, binds DOT1L and up-regulates *Hoxa* gene expression and local H3K79 methylation (Van Vlierberghe et al., 2008). Together, leukemic fusions involving Nups result in altered epigenetic and transcriptional profiles, which contribute to the pathogenesis of leukemia.

The nuclear export receptor CRM1 has indirectly been associated with human leukemias. High CRM1 expression levels correlate with aggressive human AMLs and poor prognoses (Kojima et al., 2013). Likewise, CRM1 is known to mediate nuclear export of a number of cancer-associated proteins, including BRCA1, p53, p27, and APC (Nguyen et al., 2012). When these proteins are exported out of the nucleus, they are unable to perform their nuclear functions. As these are common tumor suppressor proteins, enhanced CRM1-mediated export is thought to contribute to disease. Therefore, novel CRM1 inhibitors are being tested for efficacy in leukemias and other cancers to block nuclear export, and thus elevate nuclear levels of apoptotic proteins, such as p53 (Kojima

et al., 2013; Mutka et al., 2009; Ranganathan et al., 2012).

1.6 Summary and questions to be addressed

Chromosomal translocations involving the *CALM* and *AF10* genes are present in aggressive human leukemias (Bohlander et al., 2000). To date, it is unclear how and why the *CALM-AF10* fusion is oncogenic. AF10 is a putative transcription factor, which interacts with a number of proteins involved in gene regulation via its OM-LZ domain. AF10 may recruit genetic modifiers to the fusion through the OM-LZ domain, which is necessary and sufficient for *CALM-AF10*-mediated leukemogenesis (Deshpande et al., 2011; Okada et al., 2006). Specifically, AF10 interacts with DOT1L, the H3K79 methyltransferase, and H3K79 methylation is perturbed in *CALM-AF10*-expressing cells (Lin et al., 2009; Okada et al., 2005; Okada et al., 2006). H3K79 is globally hypomethylated across the genome, while the *HOXA* locus is locally hypermethylated and transcriptionally activated. The *HOXA* genes are critical for normal hematopoiesis, and aberrant activation of this locus results in increased proliferation and decreased apoptosis and differentiation (Lawrence et al., 1996). Therefore, mistargeting of DOT1L and activation of the *HOXA* cluster are considered to be drivers of *CALM-AF10* leukemias. However, the specific contribution of CALM and the mechanism by which CALM-AF10 causes an aberrant epigenetic and transcriptional state remain unknown.

This dissertation will examine the role of CALM in CALM-AF10-mediated leukemogenesis. First, using structure-function analyses and bone marrow transplantation assays, we will determine the structural contribution of CALM to the CALM-AF10 fusion. Finding the domain of CALM that is necessary and sufficient for leukemogenesis may provide key insights into the mechanism by which CALM-AF10 is oncogenic. Second, we will assess the function and oncogenic contribution of the minimal domain of CALM. These studies will provide a mechanistic understanding of CALM-AF10-mediated leukemogenesis and may uncover novel therapeutic targets for patients with these malignancies.

2. A CALM-derived nuclear export signal (NES) is necessary and sufficient for CALM-AF10-mediated leukemogenesis

This chapter appears in modified form in Conway A.E., Scotland, P.B., Lavau C.P., and Wechsler D.S., A CALM-derived nuclear export signal is essential for CALM-AF10-mediated leukemogenesis, *Blood* (2013).

2.1 Introduction

As discussed in Chapter 1, the t(10;11)(p13;q14) translocation gives rise to the *CALM-AF10* fusion gene and was originally identified in the U937 human monocytic cell line (Dreyling et al., 1996). Subsequently, *CALM-AF10* translocations have been identified in patients with AML, T-cell acute lymphoblastic leukemia (T-ALL), and malignant lymphoma, and are generally associated with poor prognoses (Bohlander et al., 2000; Dreyling et al., 1998). Although several breakpoints have been identified, CALM-AF10 fusion proteins consistently include most of the CALM coding sequence in-frame with a portion of AF10 that contains the octapeptide/leucine zipper (OM-LZ) protein interaction domain (Deshpande et al., 2011; Narita et al., 1999; Silliman et al., 1998). Retroviral transduction of *CALM-AF10* causes acute leukemia in a murine bone marrow transplantation model (Deshpande et al., 2006). Similarly, mice that express a *CALM-AF10* transgene develop acute leukemia at a median age of 12 months (Caudell et al., 2007). *CALM-AF10* leukemias are characterized by up-regulated expression of the *HOXA*

homeobox genes including *HOXA5*, *HOXA7*, *HOXA9*, and *HOXA10* (Caudell and Aplan, 2008; Caudell et al., 2007; Dik et al., 2005).

AF10 was first identified as a fusion partner for the *MLL* gene in patients with AML (Chaplin et al., 1995b). *AF10* encodes a nuclear protein that is a putative transcription factor and contains LAP/PHD-finger domains and nuclear localization sequences (Linder et al., 1998; Saha et al., 1995). The AF10 carboxy-terminus contains an OM-LZ domain, which has previously been shown to be necessary and sufficient for CALM-AF10-mediated leukemogenesis (Deshpande et al., 2011; Okada et al., 2005). The OM-LZ domain of AF10 interacts with various proteins including the H3K79 methyltransferase, DOT1L (Okada et al., 2005). *CALM-AF10* leukemias are marked by global hypomethylation of H3K79, while the *Hoxa* locus is H3K79 hypermethylated (Lin et al., 2009; Okada et al., 2006). Therefore, aberrant recruitment of DOT1L by the OM-LZ domain of AF10 is thought to be critical for CALM-AF10-mediated leukemogenesis (Okada et al., 2006). However, the precise mechanism by which this occurs has not yet been elucidated.

The clathrin assembly lymphoid myeloid leukemia (*CALM)* gene encodes a 652 aa protein. CALM predominantly localizes to the cytoplasm and has been shown to be necessary for the orderly progression of clathrin-mediated endocytosis (Tebar et al., 1999). Structurally, CALM contains domains that are involved in endocytosis, including an epsin N-terminal homology domain (Ford et al., 2001) and a clathrin-binding domain

in the carboxy-terminus (Scotland et al., 2012; Tebar et al., 1999). CALM has also been shown to shuttle between the cytoplasm and the nucleus where it may activate transcription, although this remains poorly understood (Vecchi et al., 2001). While perturbation of endocytosis as a result of *CALM* gene translocations has been hypothesized to play a role in leukemogenesis (Deshpande et al., 2011; Stoddart et al., 2012), the specific contributions of CALM to CALM-AF10-dependent leukemogenesis remain unclear.

In this Chapter, we perform structure-function analyses to elucidate the contributions of CALM to CALM-AF10-mediated leukemogenesis. We determined that the CALM carboxy-terminus (aa 544-553) contains a nuclear export signal (NES) that mediates cytoplasmic localization of CALM-AF10. Using *in vitro* bone marrow clonogenic assays and *in vivo* transplantation experiments, we discovered that the CALM NES is both necessary and sufficient for CALM-AF10-mediated leukemogenesis. These findings reveal a novel oncogenic mechanism by which an NES within a leukemogenic fusion protein mediates transformation.

2.2 Results

2.2.1 CALM aa 520-583 are sufficient for CALM-AF10-mediated immortalization

It has previously been determined that a region in the CALM carboxy-terminus (aa 400-648) is sufficient for CALM-AF10-mediated transformation (Deshpande et al.,

2011). To identify specific domains within this region that contribute to transformation, we performed structure-function analysis of the CALM-AF10 fusion protein. Two

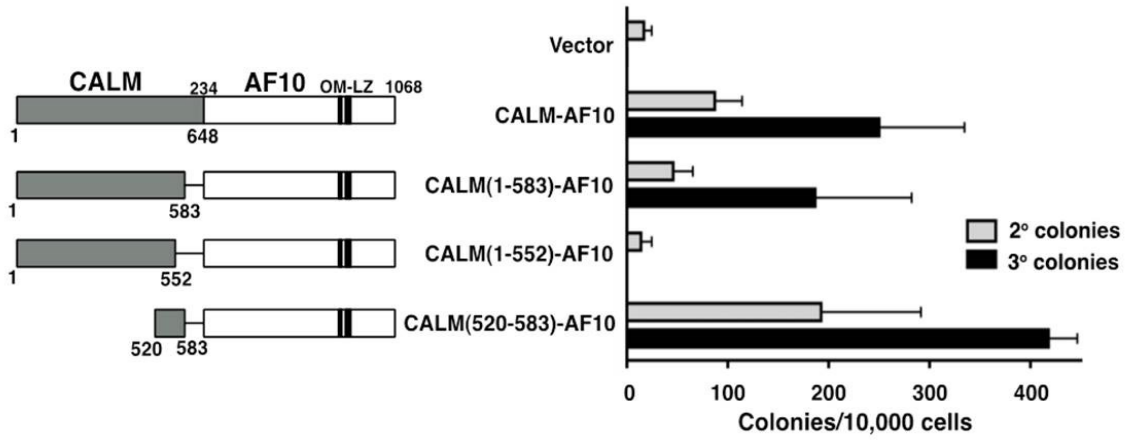

Figure 6: The region between CALM aa 520-583 is essential for CALM-AF10-mediated immortalization.

Murine bone marrow progenitor cells were transduced with retroviral constructs expressing CALM-AF10 proteins schematically shown on the left. Bar graph (right) indicates the number of colonies generated per 10,000 cells seeded in second (grey) and third (black) passage methylcellulose cultures. The mean ± SEM are shown from duplicate samples analyzed in 3 (CALM(1-552)-AF10), 4 (CALM(520-583)-AF10) or 5 (Vector, CALM-AF10, and CALM(1-583)-AF10) independent experiments.

CALM-AF10 deletion mutants were generated (CALM(1-583)-AF10 and CALM(1-552)-AF10; **Figure 6**), and we tested the ability of these truncation mutants to immortalize primary murine HPs using an *in vitro* bone marrow clonogenic assay (Lavau et al., 1997). HPs were retrovirally transduced with an empty MSCV-IRES-eGFP vector, full-length CALM-AF10, or the CALM-AF10 truncation mutants. Cells were seeded in methylcellulose, and colony formation was scored upon subsequent replatings; the presence of colonies following the third passage is indicative of extended self-renewal,

herein referred to as "immortalization" or "transformation". As shown in **Figure 6**, full-length CALM-AF10 consistently immortalizes HPs in this assay. The secondary and tertiary colonies are compact hypercellular blast-like colonies and consist of immature Mac-1^{+}/Gr-1^{+} myeloid cells and macrophages (**Figure 7**).

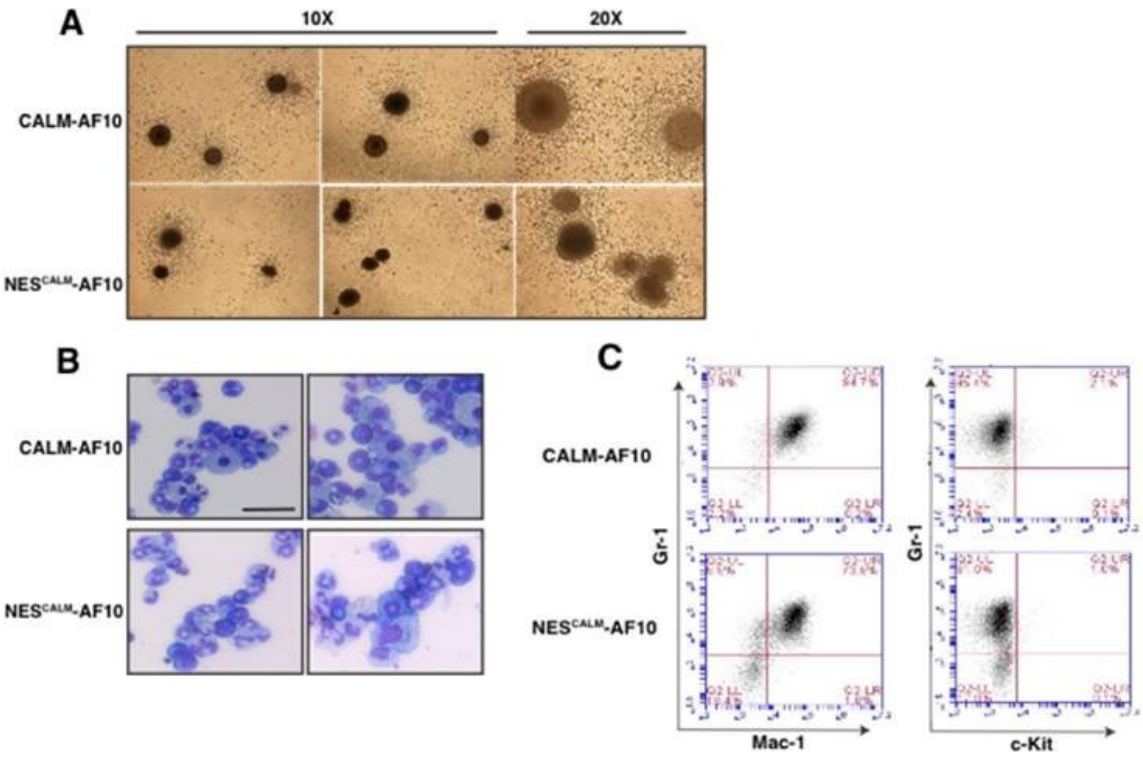

Figure 7: The morphology and immunophenotypes of CALM-AF10 and NES^{CALM}-AF10 *in vitro* methylcellulose colonies are indicative of myeloid transformation

(**A**) Colony morphology of murine bone marrow cells retrovirally transduced with CALM-AF10 (upper panels) or NES^{CALM}-AF10 (lower panels). Pictures are of representative of tertiary colonies, and were taken at 10X or 20X magnifications. (**B**) Cells from tertiary colonies were pooled, and 300,000 cells were cytospun onto coverslips to assess cellular morphology. Two representative pictures of stained CALM-AF10 (upper) or NES^{CALM}-AF10 (lower) immortalized cells are shown. Scale bar represents 40 μm for all panels. (**C**) Flow cytometric analyses of cells from CALM-AF10 (upper) or NES^{CALM}-AF10 (lower) colonies. Cells were co-stained for Gr-1 and Mac-1 (left) or Gr-1 and c-Kit (right).

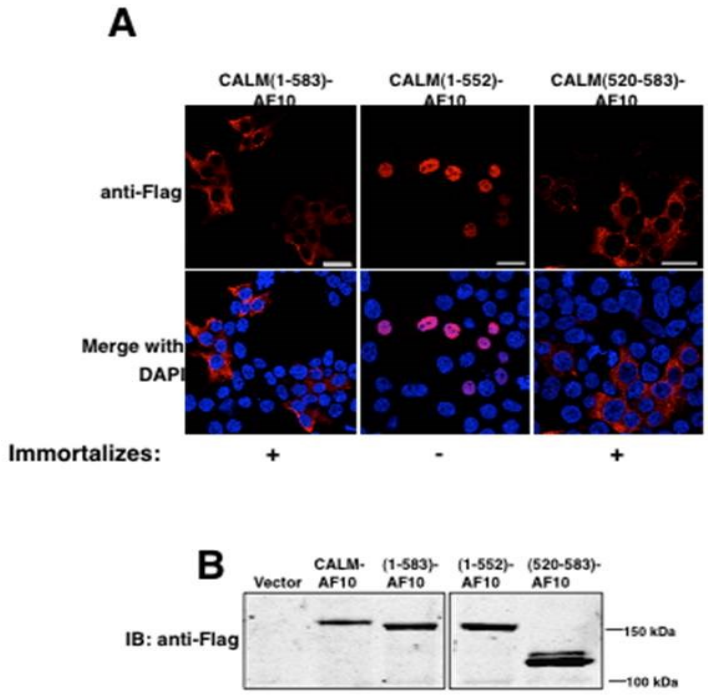

Figure 8: Transforming ability of CALM-AF10 truncation mutants correlates with cytoplasmic localization

(**A**) Confocal IF analysis of HEK293 cells transiently transfected with CALM-AF10 truncation mutants. Cell nuclei were stained with DAPI (blue). Scale bars represent 20 μm. The transforming ability of each of the mutants is shown below each panel. (**B**) Western blot of HEK293 cells transiently transfected with CALM-AF10 truncation mutants and blotted with anti-Flag antibodies.

Similar to full-length CALM-AF10, CALM(1-583)-AF10-transduced progenitors gave rise to hundreds of colonies in the third round of plating (**Figure 6**). However, CALM(1-552)-AF10-transduced cells lost their colony-forming potential in secondary passage, similar to those transduced with the empty vector control (**Figure 6**). In addition, we created a CALM-AF10 mutant that includes CALM aa 520-583 fused to AF10 (CALM(520-583)-AF10). As shown in **Figure 6**, CALM(520-583)-AF10-transduced

cells gave rise to hundreds of secondary and tertiary colonies, comparable to those seen with full-length CALM-AF10. Expression of the appropriately sized CALM-AF10 mutant proteins was verified by western blot (**Figure 8B**). From these results, we conclude that aa 520-583 of CALM are sufficient for CALM-AF10-mediated immortalization.

2.2.2 A nuclear export signal (NES) is located within CALM aa 520-583

Sequence analysis of CALM aa 520-583 revealed the presence of a putative nuclear export signal (NES) located between aa 544-553 (**Figure 10A**) (Okada et al., 2006). An NES is a hydrophobic leucine-rich amino acid sequence that is recognized by the nuclear export receptor, CRM1 (also known as XPO1). CRM1 mediates export of NES-containing proteins from the nucleus to the cytoplasm through the nuclear pore complex (**Figure 4**) (Fornerod et al., 1997). Leptomycin B (LMB) is a nuclear export inhibitor that covalently modifies CRM1 in the NES binding pocket (Kudo et al., 1998; Monecke et al., 2009). Of note, it has previously been reported that treatment with LMB causes nuclear accumulation of CALM (Vecchi et al., 2001). Because the CALM-AF10 protein localizes primarily to the cytoplasm (Okada et al., 2006), the identification of an NES motif in CALM raises the possibility that localization of the fusion protein is dependent on CRM1-mediated nuclear export. Likewise, it has been reported that fluorescent protein-fused CALM-AF10 becomes nuclear in the presence of LMB (Archangelo et al., 2006; Stoddart et al., 2012). In accord with these findings, we observed that CALM-AF10 localizes to the cytoplasm of transiently transfected HEK293 cells, and following treatment with LMB,

there is a dramatic redistribution of CALM-AF10 to the nucleus (**Figure 9A**). We next analyzed the localization of CALM-AF10 in a murine *CALM-AF10* leukemia cell line. As shown in **Figure 9B**, CALM-AF10 localizes to both the nucleus and the cytoplasm of the leukemic cells. Upon addition of LMB, CALM-AF10 becomes exclusively nuclear (**Figure 9B**). These results indicate that CALM-AF10 undergoes nucleocytoplasmic shuttling and is exported out of the nucleus in a CRM1-dependent manner.

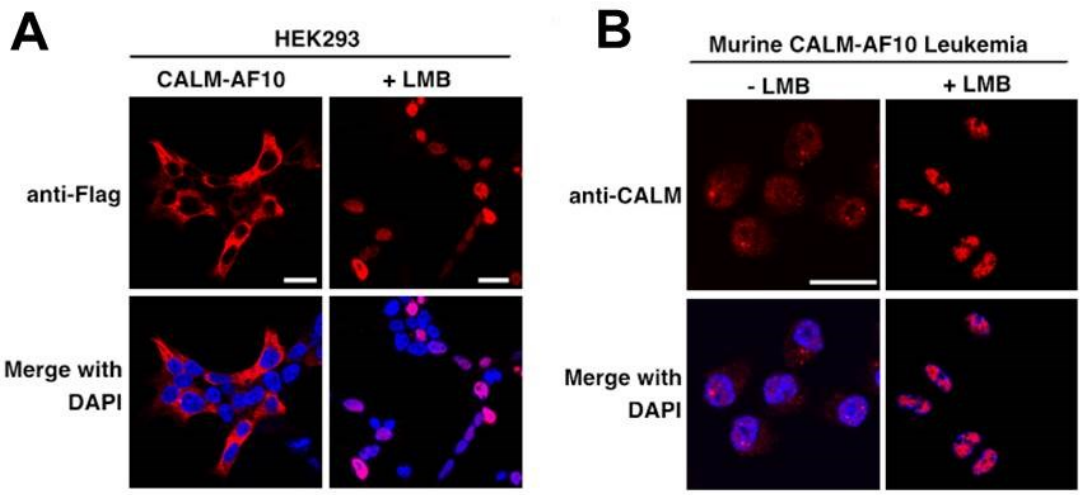

Figure 9: CALM-AF10 localizes to the cytoplasm and becomes nuclear upon treatment with Leptomycin B

(**A**) Confocal immunofluorescence (IF) analysis of HEK293 cells transiently transfected with Flag-tagged CALM-AF10 and analyzed in the absence (left) or presence (right) of LMB (10 nM, 1 hr). (**B**) Confocal IF analysis of murine *Calm*^{NULL} *CALM-AF10* leukemia cells grown in the absence (left) or presence (right) of LMB (0.1 nM, 12 hr). Cell nuclei were visualized with DAPI (blue). Scale bars represent 20 μm for all panels.

To determine the functional role of the putative CALM NES, we point-mutated conserved hydrophobic residues of the NES within full-length CALM-AF10. As shown in **Figure 10A**, 2 or 4 leucine/isoleucine residues were replaced with alanines (L544A and

L547A in CALM(mutNES1)-AF10; L551A and I553A in CALM(mutNES2)-AF10, and L544A, L547A, L551A, and I553A in CALM(mutNES1+2)-AF10). Unlike wild-type CALM-AF10, CALM(mutNES1)-AF10, CALM(mutNES2)-AF10, and CALM(mutNES1+2)-AF10 localize exclusively to the nuclei of HEK293 cells (**Figure 10B**). Similarly, when retrovirally expressed in murine hematopoietic cells, CALM-AF10 localizes predominantly to the cytoplasm, while CALM(mutNES1+2)-AF10 is exclusively nuclear (**Figure 10C**). From these findings, we conclude that CALM contains a functional NES between aa 544-553 that mediates the cytoplasmic localization of CALM-AF10.

2.2.3 The CALM NES is necessary for CALM-AF10-mediated immortalization *in vitro*

Because aa 520-583 of CALM are sufficient for immortalization (**Figure 6**) and include a CRM1-dependent NES (**Figure 10A**), we hypothesized that the transforming ability of CALM-AF10 may be dependent on the presence of the CALM NES. To test this hypothesis, CALM(mutNES)-AF10 constructs (**Figure 10A**) were retrovirally transduced into murine HPs and seeded in methylcellulose. Upon serial replatings, full-length CALM-AF10 immortalized bone marrow cells, while none of the NES point mutants resulted in colony formation (**Figure 10D**). Expression of the appropriately sized CALM-AF10 and NES point mutant proteins (180 kDa) was verified by western blot (**Figure 10D**, lower). These results suggest that a functional NES within CALM-AF10 is necessary for immortalization. Of note, the subcellular localization of the CALM-AF10 truncation mutants corroborates the link between immortalizing potential and nuclear export. When

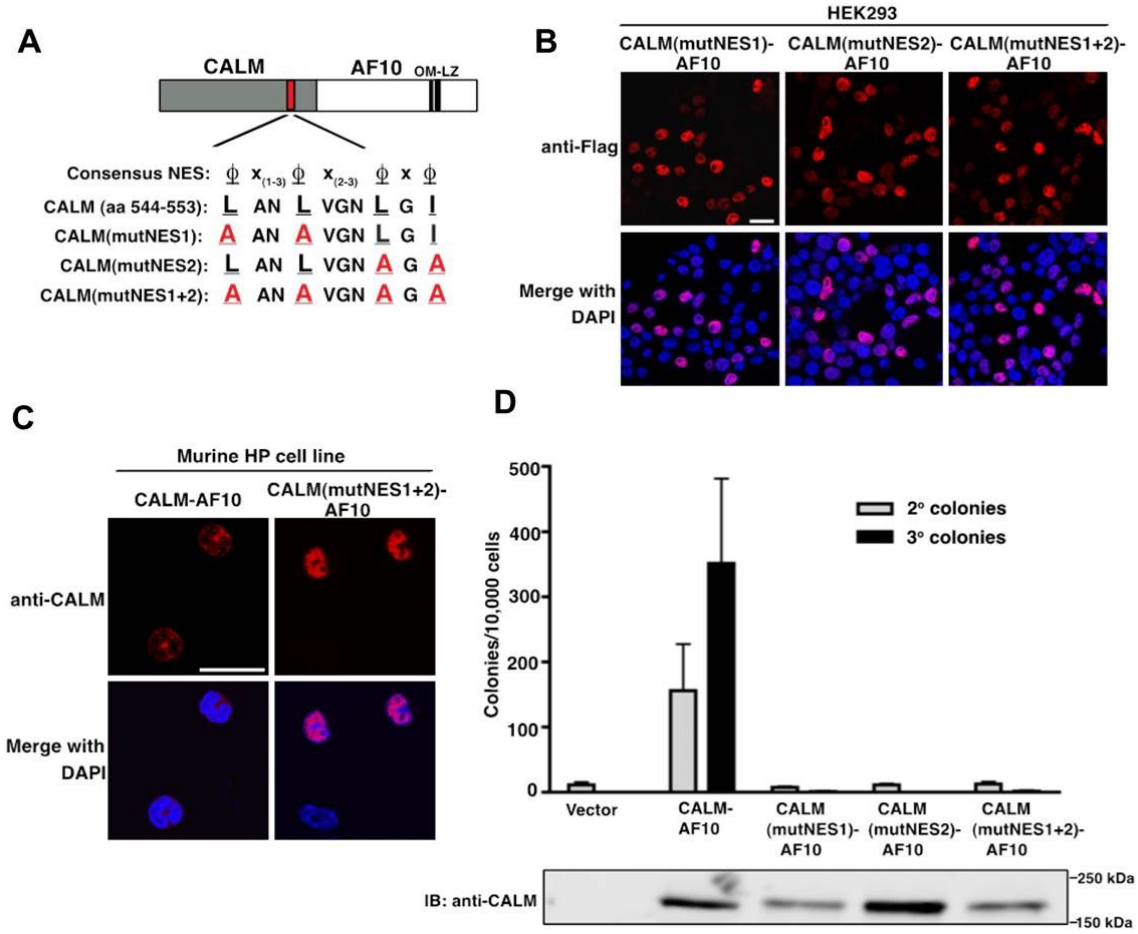

Figure 10: CALM contains a functional nuclear export signal (NES) that is necessary for CALM-AF10-depedent transformation

(**A**) Alignment of the NES within CALM (aa 544-553) and the consensus sequence of CRM1-dependent NES where ϕ represents any hydrophobic residue and x represents any amino acid. The hydrophobic aa of the CALM NES were point mutated to alanines (A) to create three mutants: CALM(mutNES1)-AF10, CALM(mutNES2)-AF10, and CALM(mutNES1+2)-AF10. (**B**) Confocal IF of HEK293 cells transiently transfected with CALM(mutNES)-AF10 mutants. (**C**) Confocal IF of MLL-ENL-immortalized *Calm*^{NULL} hematopoietic precursors retrovirally infected with Flag-tagged CALM-AF10 (left panels) or CALM(mutNES1+2)-AF10 (right panels). Cell nuclei were visualized with DAPI (blue). Scale bars represent 20 μm for all panels. (**D**) Colony forming assay of murine HPs infected with empty vector, CALM-AF10, or NES point mutants. Bars represent the number of colonies generated per 10,000 cells seeded in second (grey) and third (black) passage cultures. The mean ± SEM are shown from duplicate samples analyzed in 2 (CALM(mutNES1)-AF10), 3 (CALM(mutNES2)-AF10 and CALM(mutNES1+2)-AF10) or 6 (Vector and CALM-AF10) independent experiments. Lower panel is a western blot of HEK293 cells transfected with empty vector, CALM-AF10 and CALM(mutNES)-AF10 point mutants.

expressed in HEK293 cells, CALM(1-583)-AF10 and CALM(520-583)-AF10 are predominantly cytoplasmic, while CALM(1-552)-AF10 localizes to the nucleus (**Figure 8A**). Therefore, we conclude that the CALM NES is necessary for the cytoplasmic localization of CALM-AF10 and for *in vitro* transformation.

2.2.4 The CALM NES is sufficient for CALM-AF10-mediated immortalization *in vitro*

To determine whether the CALM NES is sufficient for CALM-AF10-mediated immortalization, we fused CALM aa 540-557 in-frame with AF10 (NES^{CALM}-AF10; **Figures 11** and **12A**). CALM aa 540-557 include the conserved CRM1-dependent NES (**Figure 10A**) with 4 aa flanking each side to maintain the structure of the NES (Henderson and Eleftheriou, 2000). When transiently transfected into HEK293 cells, NES^{CALM}-AF10 localizes predominantly to the cytoplasm, while AF10 (aa 234-1068) displays a nuclear distribution (**Figure 11A**). Following treatment with LMB, NES^{CALM}-AF10 becomes exclusively nuclear (**Figure 11A**, right). Therefore, fusion of the CALM NES to AF10 efficiently mediates CRM1-dependent nuclear export of AF10.

To test whether the CALM NES is sufficient to impart transformation potential to AF10, we examined the ability of NES^{CALM}-AF10 to immortalize murine HPs *in vitro*. It has previously been demonstrated that expression of AF10 alone does not lead to transformation (DiMartino et al., 2002). However, similar to CALM-AF10, NES^{CALM}-AF10-transduction into HPs gives rise to hundreds of secondary and tertiary colonies (**Figure**

11C) with characteristics similar to CALM-AF10 colonies (**Figure 7**). Therefore, the CALM NES fused to AF10 is sufficient for myeloid immortalization *in vitro*.

As discussed earlier, it has previously been determined that the AF10 OM-LZ domain is sufficient for CALM-AF10-mediated transformation. Fusion of full-length CALM with OM-LZ transforms murine HPs *in vitro* (data not shown and Deshpande et al. 2012). To assess whether the NES and OM-LZ domains are truly sufficient for transformation, we fused the CALM NES in frame with the carboxy-terminus of AF10 (aa 677-1068), which contains OM-LZ (termed NES-OMLZ) (**Figure 11**). This longer region of the OM-LZ domain was used because if expressed, a shorter version would be small enough to passively diffuse through the NPC. Shown in **Figure 11B**, NES-OMLZ is a cytoplasmic protein and becomes predominantly nuclear upon treatment with LMB. When retrovirally expressed and plated in methylcellulose, NES-OMLZ immortalizes murine HPs similar to both CALM-AF10 and NES^{CALM}-AF10 (**Figure 11C**). Therefore, we conclude that the CALM NES and AF10 OM-LZ domains are indeed the minimal regions necessary for CALM-AF10-mediated transformation.

2.2.5 Conserved NES protein motifs mediate nuclear export and confer immortalization potential to AF10

To determine whether the sole structural contribution of CALM is an NES, we fused NES motifs from other proteins to AF10 and assessed the immortalizing ability of the resulting chimeric proteins. Based on previous studies that characterized the functionality of multiple nuclear export consensus sequences, we chose to fuse the NES

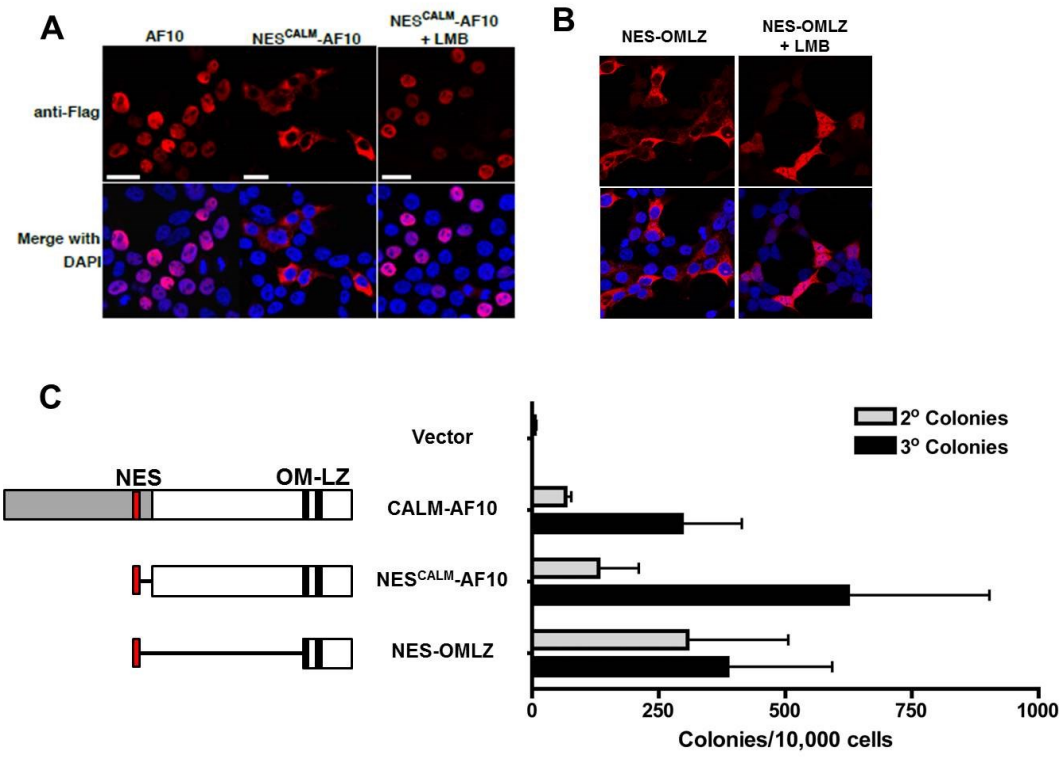

Figure 11: The CALM-derived NES and AF10 OM-LZ domain are sufficient to transform murine HPs *in vitro*

(**A**) Confocal IF analysis of HEK293 cells transfected with Flag-tagged AF10 (aa 234-1068; left) or the NES^{CALM}-AF10 fusion in the absence (middle) or presence (right) of LMB (10 nM, 1 hr). (**B**) Confocal IF of cells expressing Flag-tagged NES-OMLZ in the absence or presence of LMB. (**C**) (Left) Schematic representation of CALM-AF10, NES^{CALM}-AF10 or NES-OMLZ fusions. (Right) Colony forming assay of murine HPs infected with empty vector, CALM-AF10, or NES fusions. Bars represent the number of colonies generated per 10,000 cells seeded in second (grey) and third (black) passage cultures. The mean ± SEM are shown from duplicate samples analyzed in 3 (NES-OMLZ), 4 (CALM-AF10 and NES^{CALM}-AF10) or 7 (Vector) independent experiments.

protein motifs of ABL1, Rev (from HIV-1), APC, PKIA, and MEK1 to AF10 (**Figure 12A**) (Henderson, 2000; Henderson and Eleftheriou, 2000). As shown in **Figure 12B**, NES^{ABL1}-AF10, NES^{Rev}-AF10, NES^{APC}-AF10, NES^{PKIA}- and NES^{MEK1}-AF10 all localize to the

cytoplasm when expressed in HEK293 cells. Therefore, each of these conserved NES protein motifs mediates efficient nuclear export of AF10.

Next, we examined the ability of the NES-AF10 constructs to immortalize murine HPs *in vitro*. Primary murine HPs were retrovirally transduced with NES^{ABL1}-AF10, NES^{Rev}-AF10, NES^{APC}-AF10, NES^{PKIA}-AF10, or NES^{MEK1}-AF10 and seeded in methylcellulose. As shown in **Figure 12C**, transduction by NES^{ABL1}-AF10, NES^{Rev}-AF10, NES^{APC}-AF10, and NES^{PKIA}-AF10 fusions resulted in secondary and tertiary colonies, indicative of immortalization. These results emphasize that the fusion of an NES to AF10 is critical for the acquisition of oncogenic properties.

It should be noted that one of the heterologous NES-AF10 fusions, NES^{MEK1}-AF10 does not immortalize primary HPs *in vitro*. To further assess the structural contributions of the NES that contribute to transformation, we generated hybrids of the CALM and MEK1 NES motifs fused to AF10. As shown in Table 1, glutamate (E) residues in the third quadrant of the MEK1 NES prevent NES-AF10 from transforming murine HPs *in vitro*. Each of the hybrids efficiently mediate nuclear export of AF10 (data not shown), suggesting that there is something else important or inhibitory in this region. However, because numerous NES motifs from heterologous proteins confer transformation potential to AF10, we conclude that a CRM1-dependent NES is sufficient for CALM-AF10-mediated transformation.

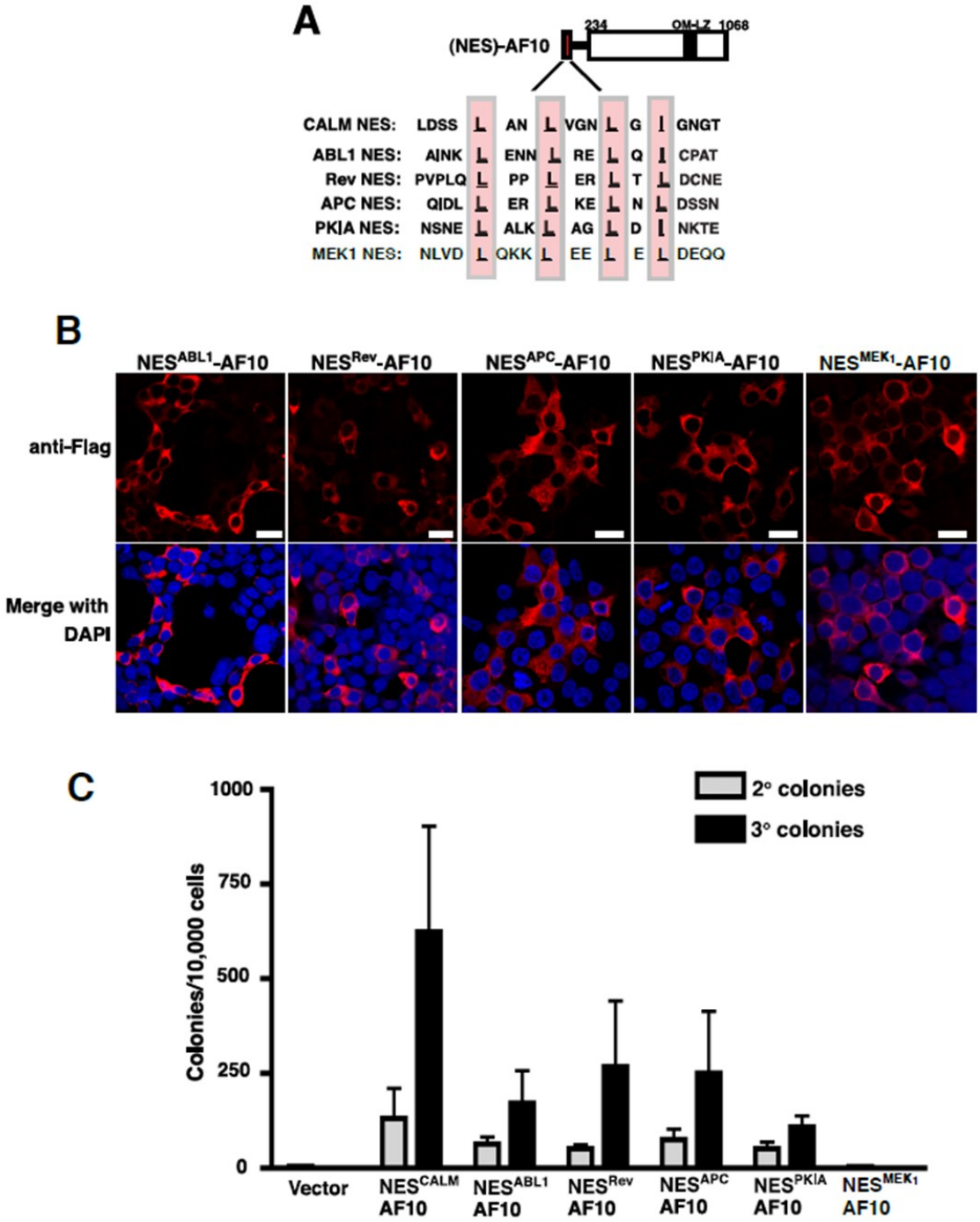

Figure 12: Fusion of conserved NES protein motifs to AF10 confers cytoplasmic localization and *in vitro* immortalization potential

Figure 12: Fusion of conserved NES protein motifs to AF10 confers cytoplasmic localization and *in vitro* immortalization potential

(**A**) Alignment of the CALM NES (aa 540-557) and the NES motifs from heterologous proteins, ABL1 (aa 1086-1103), Rev (aa 70-87), APC (aa 63-79), PKIA (aa 33-50), and MEK1 (aa 28-48) fused in-frame with AF10 (at aa 234). Key hydrophobic residues within each NES are highlighted. (**B**) Confocal IF of HEK293 cells transfected with the heterologous NES-AF10 fusions. Cell nuclei are stained with DAPI (blue). Scale bars represent 20 µm. (**C**) Colony forming assay of the NES-AF10 constructs. Bars represent the number of colonies generated per 10,000 cells seeded in second (grey) and third (black) round cultures. The mean +/- SEM are shown from duplicate samples analyzed in 3 (NES^{ABL1}-AF10 and NES^{MEK1}-AF10), 4 (NES^{CALM}-AF10, NES^{PKIA}-AF10, NES^{APC}-AF10, NES^{Rev}-AF10), or 5 (Vector) independent experiments.

Table 1: Artificial CALM-MEK1 hybrid NES motifs and transformation potential when fused with AF10

CALM NES: **LDSS-LANLV-GNLGI-GNGT** **C1 C2 C3 C4**		**MEK1 NES:** **NLVD-LQKKL-EELEL-DEQQ** **M1 M2 M3 M4**
Hybrid:	Sequence:	Transformation:
Hybrid 1 (C1-C2-M3-M4)	LDSS-LANLV-EELEL-DEQQ	**NO**
Hybrid 2 (M1-M2-C3-C4)	NLVD-LQKKL-GNLGI-GNGT	YES
Hybrid 3 (C1-M2-M3-C4)	LDSS-LQKKL-EELEL-GNGT	**NO**
Hybrid 4 (M1-C2-C3-M4)	NLVD-LANLV-GNLGI-DEQQ	YES

2.2.6 NES^{CALM}-AF10 is sufficient to induce leukemogenesis *in vivo*

To analyze the *in vivo* leukemogenic potential of NES^{CALM}-AF10, we injected CALM-AF10-, CALM(520-583)-AF10-, NES^{CALM}-AF10-, or CALM(mutNES1+2)-AF10-transduced progenitors into lethally irradiated syngeneic mice. As shown in **Figure 13A**, expression of full-length CALM-AF10 caused acute leukemia in 100% of mice (n=10) with a median survival of 122 days. CALM(520-583)-AF10 also induced leukemogenesis in 100% of mice (n=5), with a latency similar to that of CALM-AF10 (median survival of 113 days). Importantly, expression of NES^{CALM}-AF10 was sufficient to cause acute leukemia in 5 of 7 recipient mice (median survival of 250 days), while CALM(mutNES1+2)-AF10 did not cause disease up to 320 days post-transplantation (**Figure 13A**). These results strongly support the finding that the CALM NES is both necessary and sufficient for CALM-AF10-mediated leukemogenesis.

Because NES^{CALM}-AF10 leukemias developed with an extended latency than CALM-AF10 or CALM(520-583)-AF10, we further characterized each leukemia. Engraftment, as reflected by the percentage of GFP-expressing leukocytes in the peripheral blood 24 to 27 days post-transplant, occurred in all recipients (30-70% GFP, **Figure 13B**). Leukemias from mice in each cohort displayed similar characteristics, including enlarged spleens (**Figure 13C**), massive bone marrow infiltration by myeloblasts, and leukocytosis (**Figure 13D**). The vast majority of BM blasts co-expressed the myeloid antigens Mac-1 and Gr-1 while the expression of cKit and B220 were absent

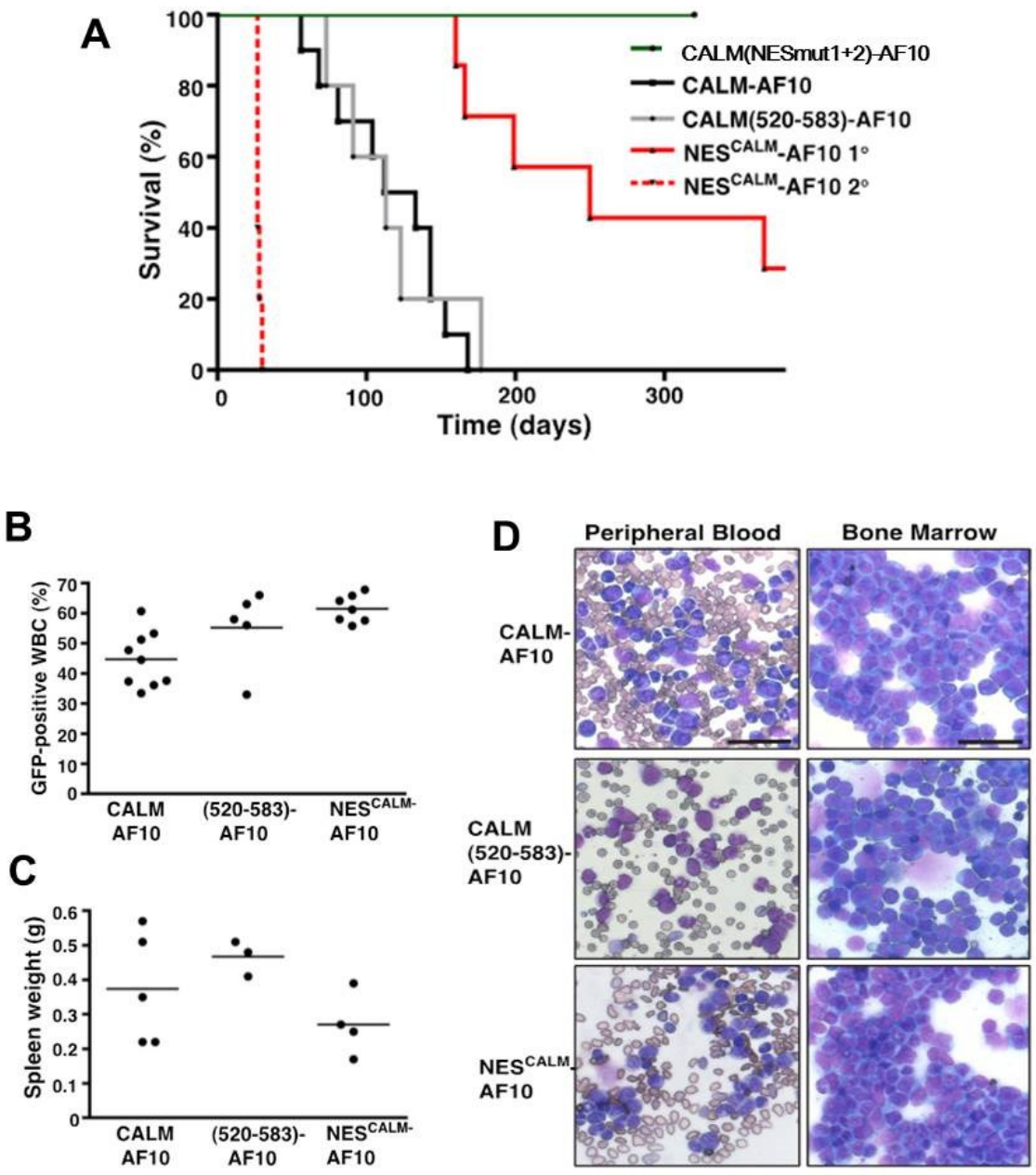

Figure 13: CALM(520-583)-AF10 and NES^{CALM}-AF10 are sufficient to induce leukemogenesis *in vivo*

Figure 13: CALM(520-583)-AF10 and NES^{CALM}-AF10 are sufficient to induce leukemogenesis *in vivo*

(**A**) Kaplan-Meier survival curve of mice transplanted with CALM(mutNES1+2)-AF10 (n=5), CALM-AF10- (n=10), CALM(520-583)-AF10- (n=5), or NES^{CALM}-AF10- (n=7) transduced bone marrow cells. All CALM(mutNES1+2)-AF10-transduced mice remained leukemia-free through the course of the study and were sacrificed at 320 days post-transplantation. The survival curve of secondary transplant mice (n=5) injected with NES^{CALM}-AF10 primary leukemias (2 primary leukemias injected to 2 or 3 mice) is depicted by a dashed red line. (**B**) Percentage of GFP-positive white blood cells (WBC) in the peripheral blood of mice measured 27 days (CALM-AF10), 25 days (CALM(520-583)-AF10) or 24 days (NES^{CALM}-AF10) post transplantation. (**C**) Spleen weights from mice with CALM-AF10, CALM(520-583)-AF10, and NES^{CALM}-AF10 leukemias measured at time of death. A normal murine spleen weighs approximately 0.09 g. (**D**) Representative peripheral blood and bone marrow smears from mice with CALM-AF10, CALM(520-583)-AF10, and NES^{CALM}-AF10 leukemias. Scale bars represent 40 μm for all panels.

or seen on a small fraction of the cells (**Figure 14**). Because NES^{CALM}-AF10 leukemias had a longer latency, we assessed their transplantability into secondary recipients. The transplanted mice died from aggressive leukemias with a median latency of 28 days (**Figure 13A**). From these results, we conclude that CALM(520-583)-AF10 and NES^{CALM}-AF10 induce acute myeloid leukemias similar to full-length CALM-AF10.

As discussed previously, CALM-AF10 leukemias are characterized by upregulation of *HOXA* cluster genes and altered H3K79 methylation. To further assess whether the leukemias induced by CALM(520-583)-AF10, and NES^{CALM}-AF10 phenocopy CALM-AF10 leukemias, *Hoxa* expression and H3K79 methylation were quantified and compared with acute myeloid leukemias induced by co-transduction of Hoxa9 and Meis1 (Hoxa9/Meis1). These leukemias serve as a control because retroviral expression of the *Hoxa9* and *Meis1* effector genes recapitulates the disease while bypassing the upstream transcriptional and epigenetic alterations regulating their expression. As shown in

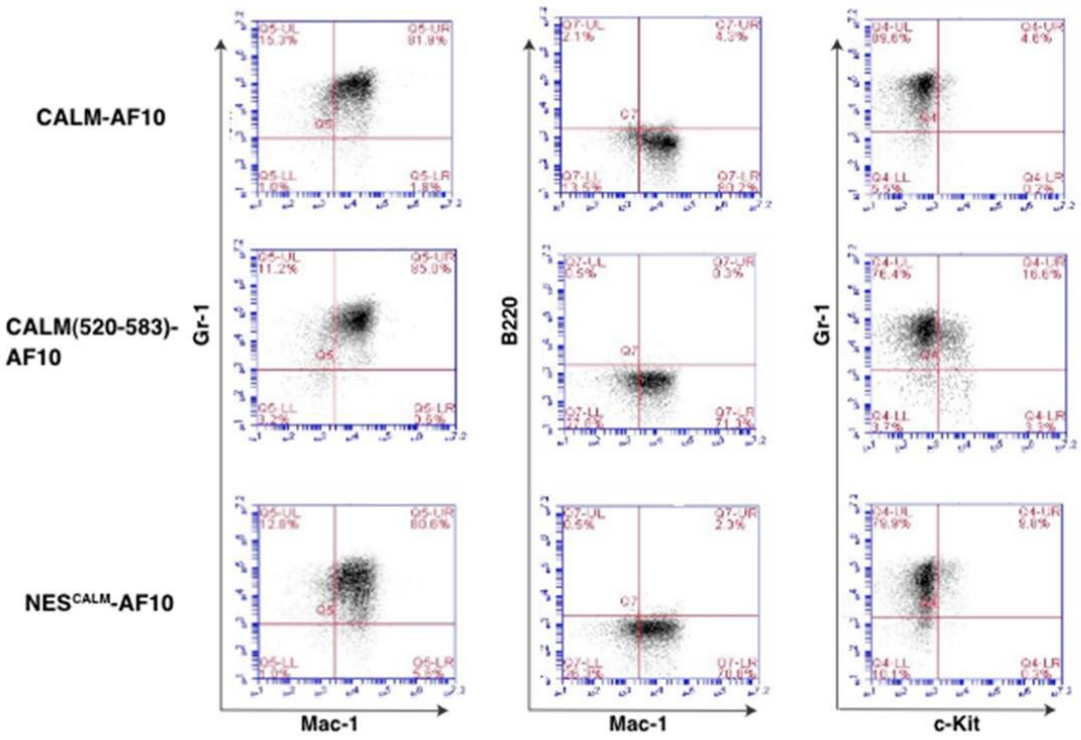

Figure 14: Primary leukemias generated by CALM-AF10, CALM(520-583)-AF10 and NES^{CALM}-AF10 have similar immunophenotypes

Flow cytometric analyses of cells from CALM-AF10 (upper row), CALM(520-583)-AF10 (middle row) or NESCALM-AF10 (bottom row) leukemic bone marrow cells. Cells were co-stained for Gr-1 and Mac-1 (left column), B220 and Mac-1 (middle column) or Gr-1 and c-Kit (right column). For each construct, data are from one leukemic mouse and are representative of at least n=3 mice.

Figure 15A, CALM-AF10, CALM(520-583)-AF10, and NES^{CALM}-AF10 leukemic cells all display reduced H3K79 di-methylation levels in comparison with Hoxa9/Meis1 leukemic cells (reduction of 55%, 30%, and 40%, respectively).

In contrast to Hoxa9/Meis1 leukemic cells, CALM-AF10, CALM(520-583)-AF10 and NES^{CALM}-AF10 leukemic cells all display elevated *Hoxa5*, *Hoxa7*, *Hoxa10*, and *Hoxa11*

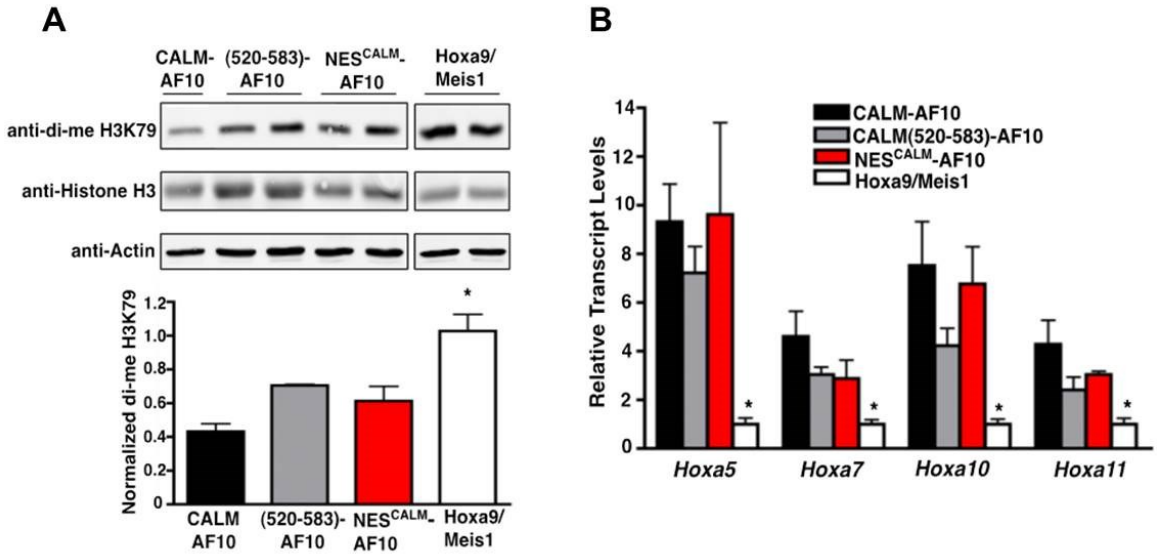

Figure 15: CALM(520-583)-AF10 and NES^{CALM}-AF10 leukemia cells have reduced global H3K79 methylation and elevated *Hoxa* gene expression

(**A**) Representative western blots of di-methylated H3K79, Histone H3, and Actin protein levels in separate CALM-AF10 (shown is 1 sample out of 3) , CALM(520-583)-AF10 (shown are 2 samples out of 3), NES^{CALM}-AF10 (shown are 2 samples out of 3), and Hoxa9/Meis1 (shown are 2 samples out of 3) leukemias. Quantification data represent the mean ± SEM of di-me H3K79 values normalized to Histone H3 from separate leukemias (n=3 for each). Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. Only Hoxa9/Meis1 was found to be statistically different from CALM-AF10; **P*<.05. (**B**) *Hoxa* transcript levels were obtained by Real Time RT-PCR and normalized to housekeeping genes GAPDH and β2M and then to Hoxa9/Meis1 leukemic cells by the ΔΔCt method. Results are shown as mean ± SEM compiled from 3 (CALM(520-583)-AF10, NES^{CALM}-AF10) or 4 (CALM-AF10, and Hoxa9/Meis1) separate leukemias. Statistical analysis was performed by one-way ANOVA for each *Hoxa* gene followed by Dunnett's multiple comparison test. Only Hoxa9/Meis1 was found to be statistically different from CALM-AF10; **P*<.05.

transcript levels (**Figure 15B**). Therefore, the CALM NES fused to AF10 is sufficient to up-regulate *Hoxa* cluster expression. We next assessed the epigenetic status of the *Hoxa* locus in the CALM-AF10 and NES^{CALM}-AF10 leukemic cells. Both CALM-AF10 and NES^{CALM}-AF10 leukemic cells exhibit enriched H3K79 di-methylation on *Hoxa* gene promoters (**Figure 16A**). We observed a similar pattern of H3K4 tri-methylation, a mark of active

transcription, on the *Hoxa* promoters in CALM-AF10 and NES^{CALM}-AF10 cells (**Figure 16B**). Therefore, the fusion of the CALM NES to AF10 phenocopies the aberrant epigenetic and transcriptional profile observed in *CALM-AF10* leukemias.

2.2.7 Inhibition of CRM1 impairs viability of human CALM-AF10 leukemia cells

We have shown that mutation of the CALM NES results in nuclear accumulation of CALM-AF10 and impairs leukemogenic transformation. We therefore hypothesized that treatment with a nuclear export inhibitor (NEI) could prevent CALM-AF10 from interacting with the nuclear export receptor CRM1 and potentially hinder cell proliferation. To test the sensitivity of CALM-AF10-expressing cells to NEIs, we compared the viability of *CALM-AF10*-positive (P31/Fujioka (Narita et al., 1999) and U937) and *CALM-AF10*-negative (K562 and THP-1) human leukemia cell lines in the presence of LMB. As determined by RT-PCR, P31/Fujioka and U937 cells both express the *CALM-AF10* transcript, while THP-1 and K562 cells do not (**Figure 17A**). Of note, transcripts of differing sizes were amplified from the P31/Fujioka and U937 cells, consistent with the alternate *CALM-AF10* fusion breakpoints in these cell lines (Narita et al., 1999). Cells were seeded at fixed densities, and cell viability was measured in the presence of varying concentrations of LMB. *CALM-AF10*-positive cells showed a greater sensitivity to 0.5 and 1 nM LMB after 17 hr of treatment, with approximately 40% viable cells remaining compared with 80-100% *CALM-AF10*-negative cells (**Figure 17B**). The same effect was observed after 42 hr of LMB treatment: 1-11% of the *CALM-AF10*-positive

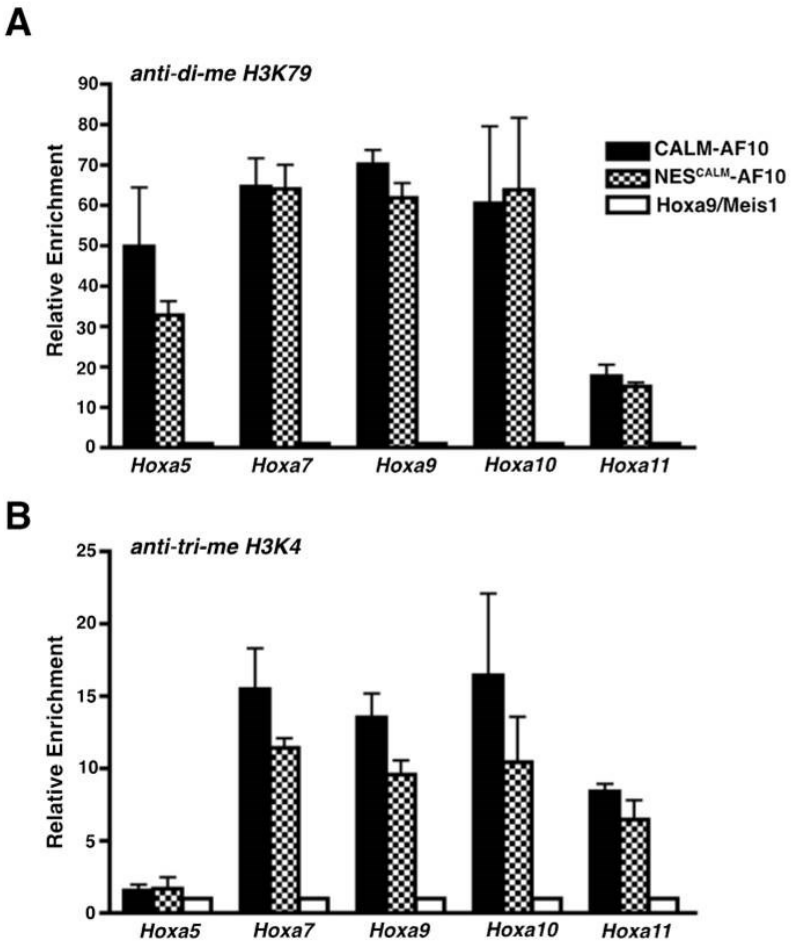

Figure 16: H3K79 and H3K4 are hypermethylated on the *Hoxa* locus in CALM-AF10 and NES^{CALM}-AF10 leukemias.

ChIP analysis of (**A**) di-methylated H3K79 and (**B**) tri-methylated H3K4 in the promoter regions of the *Hoxa* cluster genes. *Hoxa* amplification was measured by Real Time PCR as a percent of input then normalized to Hoxa9/Meis1 control leukemic cells. Black bars represent CALM-AF10, checkered bars are NES^{CALM}-AF10 and white bars are Hoxa9/Meis1 leukemias. Results are shown as mean ± SEM compiled from 3 separate leukemias.

cells were viable, while 35-45% of the *CALM-AF10*-negative cells were viable in the presence of 0.5 or 0.1 nM LMB (**Figure 17C**). These results indicate that *CALM-AF10*-expressing human leukemia cells display increased sensitivity to inhibition of CRM1, and support investigation of the therapeutic utility of NEIs in *CALM-AF10* leukemias.

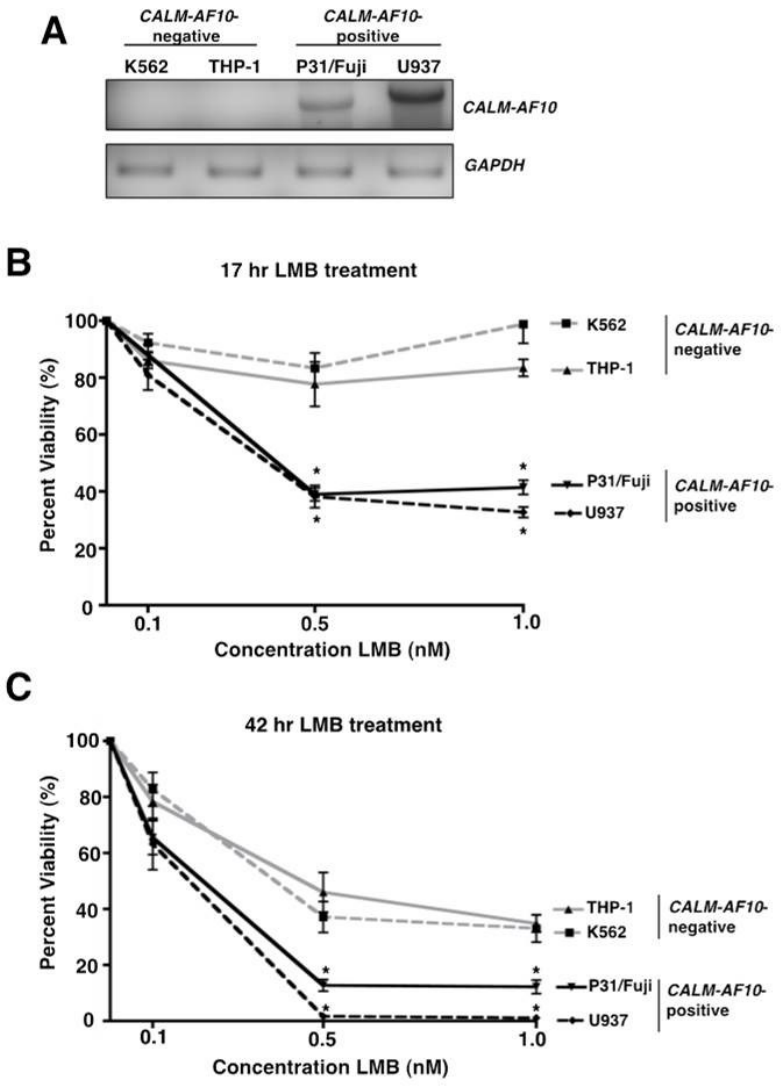

Figure 17: Blocking nuclear export decreases viability of human *CALM-AF10* leukemia cell lines

(**A**) Confirmation of expression of the *CALM-AF10* transcript in P31/Fujioka (P31/Fuji) and U937 cells by RT-PCR. GAPDH serves as a control for equal input of RNA for RT-PCR. (**B-C**) *CALM-AF10*-positive (P31/Fujioka and U937) and *CALM-AF10*-negative (K562 and THP-1) cell lines were grown in the presence of increasing concentrations of LMB (0, 0.1, 0.5, 1 nM) for 17 hr (**B**) or 42 hr (**C**). The number of viable cells was determined by flow cytometry and is shown as a percent of untreated for each cell line. Results are shown as mean ± SEM from 3-6 independent experiments. The viabilities of both P31/Fuji and U937 cell lines are statistically different from either K562 or THP-1 at 0.5 and 1 nM LMB, as determined by two-way ANOVA followed by Bonferroni's Multiple Comparison Test; **P*<.05.

2.3 Discussion

Fusion proteins have been shown to mediate hematopoietic oncogenesis by a variety of mechanisms. Some chimeric fusion proteins involve DNA-binding transcription factors (e.g. AML1-ETO or NUP98-HOXA9) (Gardini et al., 2008; Yassin et al., 2009), cofactors (e.g. MOZ-rearrangements or MN1-TEL) (Buijs et al., 2000; Katsumoto et al., 2008), or chromatin modifying proteins (e.g. MLL-fusions) (Marschalek, 2011), and co-opting of the properties of the fusion partners leads to aberrant transcriptional activity. Other fusion partners provide homo-oligomerization domains that enable dimerization of the fusion protein, resulting in enhanced or novel activities (such as PML-RARα or some MLL-fusions) (Lin and Evans, 2000; Martin et al., 2003; So et al., 2003). Several endocytic proteins (e.g. CALM, EPS15, or CLTC) are targeted by chromosomal translocations, and perturbation of endocytosis has been proposed as a mechanism of leukemogenesis (Crosetto et al., 2005; Lanzetti and Di Fiore, 2008). The well-documented involvement of CALM in endocytosis (Miller et al., 2011; Tebar et al., 1999) and its potential transcriptional role in the nucleus (Vecchi et al., 2001) led us to assume that either (or both) of these functions could be involved in the oncogenicity of CALM-AF10. However, our structure-function analysis of CALM revealed an unexpected and novel mechanism of leukemic transformation that is dependent on the presence of a nuclear export signal.

Using bone marrow immortalization and transplantation assays, we have demonstrated that an NES within CALM (aa 544-553) is both necessary and sufficient for CALM-AF10-mediated leukemogenesis. These findings complement the observations of Deshpande et al. (2011) who reported that CALM aa 400-648 are sufficient for CALM-AF10-driven transformation. These authors attributed their findings to the presence of a clathrin-binding domain in the carboxy-terminus of CALM, and concluded that disruption of endocytosis may be a mechanism by which CALM-AF10 is oncogenic. We have recently determined that the CALM clathrin-binding domain spans aa 583-652 at the extreme carboxy-terminus, which is distinct from the NES (Scotland et al., 2012). Similarly, Stoddart et al. (2012) have shown that endocytosis is not perturbed in CALM-AF10 cells. Instead, these authors suggested that homo-oligomerization of CALM-AF10 may mediate its transformation potential, based on the ability of the last 55 aa of CALM within CALM-AF10 (aa 593-648) to facilitate dimerization. Our observation that CALM aa 540-557 or NES motifs from heterologous proteins are sufficient to confer transforming potential to AF10 led us to conclude that a CRM1-dependent NES represents the functional contribution of CALM in CALM-AF10-mediated leukemogenesis and is distinct from the motifs of CALM involved in endocytosis or oligomerization.

We have shown that fusion of the 18 aa CALM NES in-frame with AF10 (NES^{CALM}-AF10) is sufficient to immortalize murine progenitor cells *in vitro* and to induce leukemogenesis *in vivo*. It is important to note that NES^{CALM}-AF10 leukemias display

prolonged latencies, while a larger portion of CALM spanning the NES fused to AF10 (CALM(520-583)-AF10) fully recapitulates the rapid onset of *CALM-AF10* leukemias (**Figure 13A**). It is possible that the longer CALM(520-583)-AF10 allows for proper folding of the NES and/or contributes to its overall stability. It is also possible that CALM(520-583)-AF10 contains an additional domain(s) that contributes to leukemogenesis. For example, CALM contains a putative transcriptional activation domain (TAD) that spans aa 408-572 (Archangelo et al., 2006). Additional residues of the TAD present in CALM(520-583)-AF10 could recruit transcriptional complexes or directly interact with DNA regulatory regions. Because the function of the CALM TAD remains unknown, additional studies are required to elucidate its potential contribution to CALM-AF10-dependent leukemogenesis. Importantly, our demonstration that NES^{CALM}-AF10 induces leukemogenesis *in vivo* suggests that the CALM NES is sufficient for CALM-AF10-mediated oncogenicity.

CALM-AF10 leukemias are characterized by a global reduction in H3K79 methylation, and this phenotype may contribute to leukemogenesis by increasing chromosomal instability (Lin et al., 2009). Here, we show that like CALM-AF10, both CALM(520-583)-AF10 and NES^{CALM}-AF10 leukemias display a global loss of H3K79 methylation. It has previously been proposed that CALM-AF10 acts in a dominant negative manner on endogenous AF10 to mediate global H3K79 hypomethylation (Lin et al., 2009). Because the OM-LZ domain of AF10 is necessary and sufficient for CALM-

AF10-mediated transformation, CALM-AF10 could alter the subcellular localization of the OM-LZ binding partner DOT1L. The relative exclusion of DOT1L from the nucleus may explain the global loss of H3K79 methylation observed in CALM-AF10 expressing cells. Additional studies are necessary to determine the effect of nuclear export on DOT1L and subsequent downstream H3K79 methylation.

In contrast to a global reduction in H3K79 methylation, the *HOXA* homeobox genes are H3K79 hypermethylated and transcriptionally up-regulated in CALM-AF10 leukemias. Our finding that both H3K79 hypermethylation and *Hoxa* gene upregulation are found in NES^{CALM}-AF10 leukemias is intriguing and suggests a potential role of the CALM NES in these aberrations. Because the CALM-derived NES mediates nuclear export of CALM-AF10, the effects on the *Hoxa* loci may be indirect, possibly by mislocalizing important transcriptional/epigenetic regulators to the cytoplasm. However, Okada et al. (2006) showed that CALM-AF10 binds the *Hoxa5* locus by ChIP. It is possible that CALM-AF10 directly binds chromatin as it undergoes nucleocytoplasmic shuttling. Likewise, the nuclear pore has been characterized as a site of active gene transcription in eukaryotes (Brown and Silver, 2007). Therefore, it is conceivable that the CALM-derived NES targets CALM-AF10 to the nuclear periphery (via CRM1), where *Hoxa* locus-specific gene activation may occur. Additional experiments are necessary to elucidate the role of the NES in mediating up-regulation of the *Hoxa* locus in these leukemias.

Aggressive hematopoietic malignancies harboring *CALM-AF10* translocations are seen in both pediatric and adult patients and are associated with poor prognoses. Improving the outcome of *CALM-AF10* leukemias depends on the development of targeted therapies with improved efficacy and reduced toxicity. Based on our discovery that immortalization by *CALM-AF10* is dependent on a CALM NES, we hypothesize that nuclear export inhibitors represent an innovative approach to selectively target these malignancies. Importantly, we have shown that human *CALM-AF10*-expressing leukemic cells are more sensitive to treatment with LMB than *CALM-AF10*-negative lines (**Figure 17B-C**). Additionally, blocking nuclear export has been proposed as a therapeutic strategy against many types of cancer based on diverse mechanisms of action, such as preventing signaling through p53 or NFκB (Mutka et al., 2009; Takeda et al., 2010). In fact, LMB was tested as an anti-cancer agent in phase I clinical trials, but due to dose-limiting toxicity, it was not pursued further (Newlands et al., 1996). Despite concerns about toxicity, targeting nuclear export remains an attractive possibility, and new, more potent and less toxic agents are being tested (Ranganathan et al., 2012). Therefore, blocking CRM1-dependent nuclear export is a potential therapeutic possibility for patients with CALM-AF10 leukemias. In addition, it may be beneficial to develop a small molecule inhibitor specifically directed against the NES of CALM to selectively block nuclear export of the CALM-AF10 oncoprotein.

In summary, a CALM-derived NES is both necessary and sufficient to confer transformation potential to AF10. We hypothesize that CRM1 inhibitors may represent a novel therapeutic approach for patients with *CALM-AF10* leukemias. Mechanistic studies to address how the CALM NES imparts transformation potential to AF10 are underway and will be discussed in the next Chapter of this dissertation.

3. Mechanisms by which the CALM NES imparts transformation potential to CALM-AF10

This chapter appears in modified form in Conway A.E., Scotland P.B., Lavau C.P., and Wechsler D.S., A CALM-derived nuclear export signal is essential for CALM-AF10-mediated leukemogenesis, *Blood* (2013).

3.1 Introduction

In Chapter 2, we determined that a nuclear export signal (NES) within CALM is both necessary and sufficient for CALM-AF10-mediated leukemogenesis (**Figure 18A**). It has previously been determined that the OM-LZ protein interaction domain is the critical oncogenic component within AF10. Together, these structure-function analyses have demonstrated that the leukemogenic activity of CALM-AF10 is due to the function of two regions, the CALM NES and the AF10 OM-LZ domain. The identification of the domains that are critical for the transformation potential of CALM-AF10 may provide insights into the mechanisms by which CALM-AF10 causes leukemia. By understanding these oncogenic mechanisms, potential therapeutic targets may be identified.

In this Chapter, we extend our observations of the key structural components of *CALM-AF10* to elucidate the molecular mechanisms by which this gene fusion is leukemogenic. Specifically, we focus on understanding how the CALM-derived NES imparts transformation potential to AF10.

First, we will assess the hypothesis that aberrant cytoplasmic localization of AF10 is on its own oncogenic (**Figure 18B**). Irregular cytoplasmic localization of a nuclear

protein may affect multiple cellular pathways and drive leukemia by loss-of-function or gain-of-function mechanisms. Interestingly, this type of abnormality has been documented in acute leukemias with *NPM1* mutations. Point mutations within the *NPM1* gene result in ectopic formation of an NES, and cytoplasmic presence of NPM1 has been directly associated with leukemogenesis (Falini et al., 2007; Falini et al., 2006). As shown in Chapter 2 (**Figures 11-12**), fusions of NES motifs to AF10 result in steady-state cytoplasmic localization and transformation of murine HPs *in vitro*. Here, we assess whether expression of a truncated version of AF10 that cannot localize to the nucleus results in transformation *in vitro*.

Second, we will examine the hypothesis that nuclear export of AF10 mislocalizes an OM-LZ binding partner (**Figure 18C**). Although cytoplasmic at steady-state, both CALM-AF10 and NES^{CALM}-AF10 constantly shuttle between the nucleus and the cytoplasm. Abnormal nucleocytoplasmic shuttling of the OM-LZ protein interaction motif could result in displacement of a binding partner. Specifically, OM-LZ interacts with the DOT1L H3K79 histone methyltransferase, and *CALM-AF10* leukemias are characterized by a global loss of H3K79 methylation. Therefore, we theorize that nuclear export of AF10 displaces DOT1L and results in a loss-of-function for DOT1L's methyltransferase activity in the nucleus. To test this hypothesis, we will assess whether the NES is critical for CALM-AF10-mediated H3K79 hypomethylation, and we will elucidate the localization of DOT1L in the presence of CALM-AF10. Because H3K79

hypomethylation has been associated with chromosomal instability (Lin et al., 2009), results from these experiments may provide key insights into an indirect mechanism by which CALM-AF10 contributes to leukemogenesis.

Finally, we will analyze the importance of the NES in mediating up-regulation of the *Hoxa* cluster genes (**Figure 18D**). As discussed in Chapters 1 and 2, *HOXA* cluster loci are locally H3K79 hypermethylated and transcriptionally up-regulated in *CALM-AF10* leukemias. Controlled expression of *HOXA* genes is critical for normal hematopoiesis, and aberrant activation of these transcription factors is a driving factor in acute leukemogenesis. Therefore, the *HOXA* genes are considered to be critical oncogenic components of *CALM-AF10* leukemias; however, the mechanism by which CALM-AF10 up-regulates these genes remains unclear. In Chapter 2, we demonstrated that NES^{CALM}-AF10 leukemias phenocopy the transcriptional and epigenetic profiles of CALM-AF10 leukemias, suggesting that the NES motif is sufficient for these abnormalities (**Figures 15-16**). Here, we will assess the necessity of the CALM-derived NES for local H3K79 hypermethylation and activation of the *Hoxa* locus. We hypothesize that the CALM-CRM1 interaction is critical for targeting CALM-AF10 to *Hoxa* chromatin, and we will test the utility of nuclear export inhibitors to interfere with these transcriptional effects.

The goal of this Chapter is to provide novel mechanistic insights into the pathogenesis of *CALM-AF10* leukemias. These studies will extend our finding that the

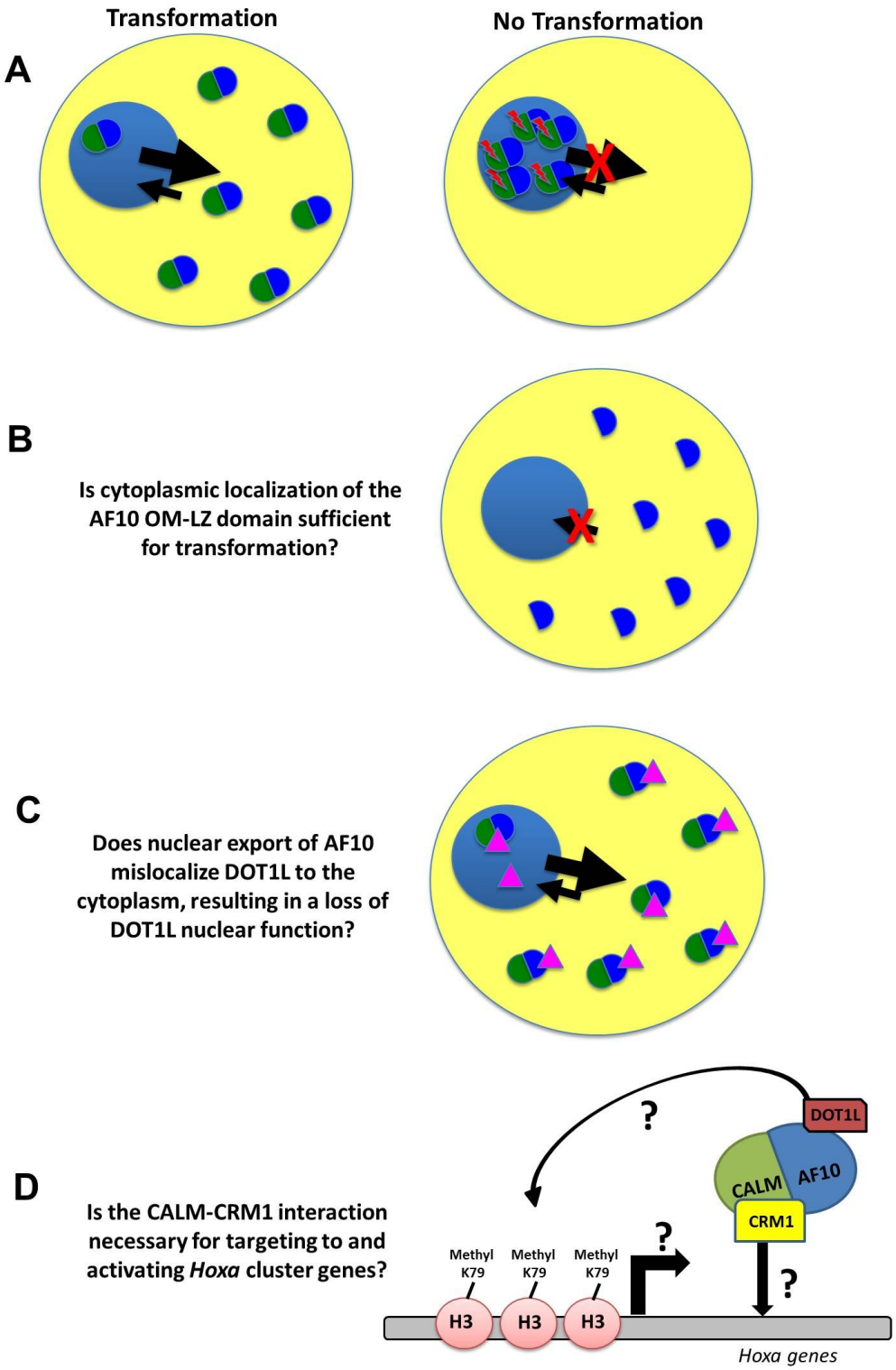

Figure 18: Possible mechanisms by which the CALM NES contributes to leukemogenesis

Figure 18: Possible mechanisms by which the CALM NES contributes to leukemogenesis

(**A**) Diagrammatic representation of findings from Chapter 2. CALM-AF10 (green-blue circles) is predominantly cytoplasmic due to CRM1-mediated nuclear export (left). When the CALM NES is point mutated (red bolt), CALM-AF10 is exclusively nuclear and cannot transform murine bone marrow cells. (**B**) Because cytoplasmic localization of CALM-AF10 correlates with transformation potential, we will assess whether cytoplasmic retention of AF10 (blue half-circles) is sufficient for transformation of HPs *in vitro*. (**C**) CALM-AF10 shuttles between the nucleus and the cytoplasm (black arrows). We will examine whether CALM-AF10 mislocalizes the AF10 binding partner, DOT1L (pink triangle) to the cytoplasm. CALM-AF10 cells are globally H3K79 hypomethylated, and loss of nuclear DOT1L may contribute to this phenotype. (**D**) CALM-AF10-expressing cells have local H3K79 hypermethylation and transcriptionally activated *Hoxa* cluster genes. We will assess whether the NES is necessary for binding to *Hoxa* cluster genes leading to elevated H3K79 methylation and transcriptional activation. Specifically, we will explore whether the CALM-CRM1 interaction mediates these leukemogenic phenotypes.

CALM-derived NES is critical for leukemogenesis and will elucidate the consequences of fusion of the NES to AF10. Little is known about the oncogenic mechanism by which CALM-AF10 is leukemogenic, and further mechanistic insight may help to elucidate therapeutic targets for patients with these malignancies. In addition, the genetic and epigenetic profiles of *CALM-AF10* leukemias phenocopy those of *MLL* leukemias, suggesting that they may share a common oncogenic mechanism. Therefore, insights into the molecular mechanisms underlying CALM-AF10 leukemias may have broad implications for other types of aggressive hematopoietic malignancies.

3.2 Results

3.2.1 Cytoplasmic localization of AF10 is not sufficient for transformation

AF10 is a putative transcription factor with numerous nuclear localization sequences (NLSs) (**Figures 3** and **19**) that mediate nuclear localization of AF10. As shown in Chapter 2, fusion of a CRM1-dependent NES to AF10 mediates nuclear export of AF10, resulting in steady-state cytoplasmic localization. In addition, the NES motif is sufficient to confer oncogenic potential to AF10. Therefore, we have observed that leukemic transformation correlates with cytoplasmic localization of AF10. Based on these observations, we hypothesized that cytoplasmic localization of AF10, specifically the OM-LZ domain, may be sufficient for transformation of HPs.

To test whether cytoplasmic localization of AF10 is sufficient for transformation, we generated a version of AF10 that does not localize to the nucleus, which we called "cytoAF10" (**Figure 19A**). To generate this mutant, a start site and Flag-tag were inserted immediately following the second NLS within AF10 by PCR, resulting in a truncated version of AF10 that is missing the 2 main NLS motifs (**Figure 19A**). cytoAF10 is 598 aa, corresponding to a ~66 kDa protein. Therefore, it is too large to passively diffuse through the nuclear pore complex (Gorlich and Mattaj, 1996). As shown in **Figure 19B**, cytoAF10 localizes to the cytoplasm, and its localization does not change upon treatment with LMB. Although we cannot rule out the possibility that cytoAF10 is exported out of the nucleus in a CRM1-independent manner, its steady state localization is similar to NES^{CALM}-AF10

(**Figure 11A**). We assessed whether cytoAF10 is sufficient to transform murine HPs using an *in vitro* bone marrow transformation assay. As shown in **Figure 19B**, while CALM-AF10 gives rise to many secondary and tertiary colonies, cytoAF10 does not transform murine progenitors. From these results, we conclude that ectopic cytoplasmic expression of the OM-LZ region of AF10 is not sufficient for transformation. Therefore, either nucleocytoplasmic shuttling or fusion of a CRM1-dependent NES sequence is necessary to impart transformation potential to AF10, although these options are not mutually exclusive.

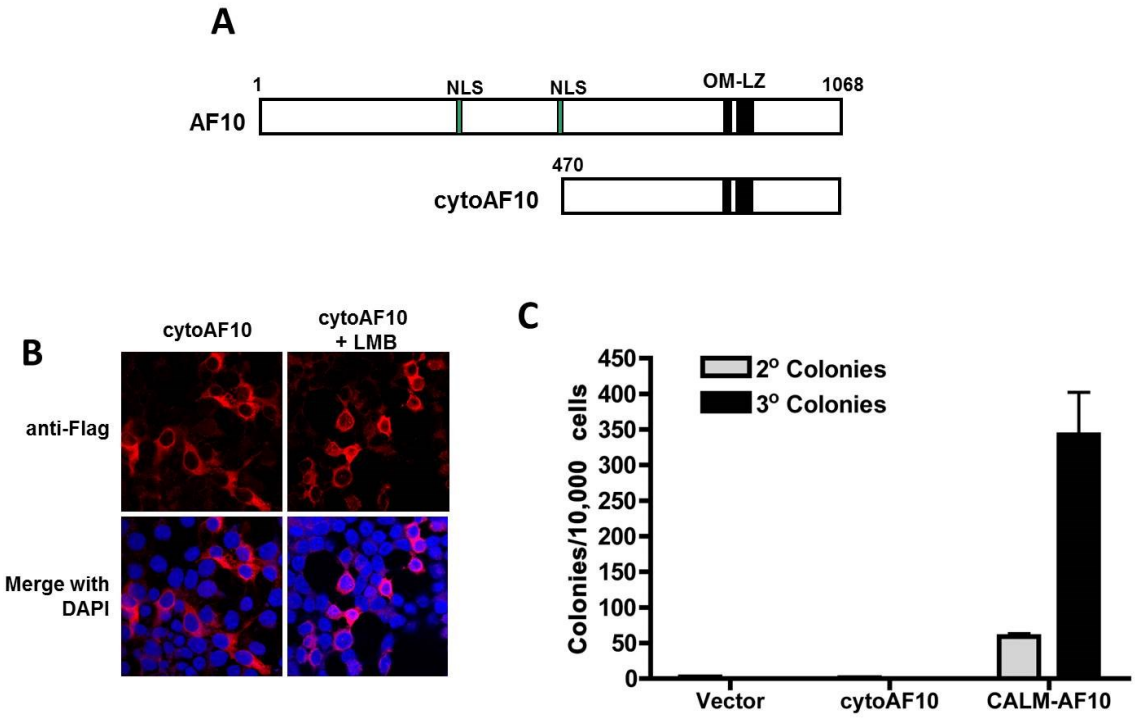

Figure 19: Ectopic cytoplasmic presence of AF10 is not sufficient for transformation

Figure 19: Ectopic cytoplasmic presence of AF10 is not sufficient for transformation
A) Schematic representation of AF10 and the conserved nuclear localization sequences (NLS). AF10 was truncated following the second NLS (aa 470) and termed cytoAF10. (**B**) Confocal IF analysis of HEK293 cells transiently transfected with cytoAF10 alone or with 10 nM LMB for 24 hr. Cell nuclei were stained with DAPI (blue). (**C**) Colony forming assay of murine HPs infected with empty vector, cytoAF10, or CALM-AF10. Bars represent the number of colonies generated per 10,000 cells seeded in second (grey) and third (black) round cultures. The mean +/- SEM are shown from duplicate samples analyzed in 2 (cytoAF10 and CALM-AF10) or 4 (vector) independent experiments.

3.2.2 Nuclear export of CALM-AF10 mislocalizes DOT1L, resulting in global loss of H3K79 methylation

Cytoplasmic accumulation of AF10 is not sufficient for transformation, which suggests that fusion to the CALM-derived NES is necessary. Characteristically, an NES interacts with the CRM1 nuclear export receptor, resulting in nuclear export through the nuclear pore complex in a Ran-GTP-dependent manner (**Figure 4**). Therefore, we hypothesized that fusion of the NES to AF10 and subsequent nuclear export contributes to the transformed phenotype.

As discussed previously, *CALM-AF10* leukemias are marked by a global loss of H3K79 di-methylation. DOT1L is the sole H3K79 histone methyltransferase, and to date, no demethylase has been identified. Therefore, global H3K79 hypomethylation is thought to be the result of loss of DOT1L function. AF10 interacts with DOT1L via the OM-LZ domain, and expression of CALM-AF10 missing the OM-LZ domain (CALM-AF10ΔOMLZ) does not cause H3K79 hypomethylation (Lin et al., 2009). Thus, through a

direct interaction with CALM-AF10, DOT1L is dissociated from chromatin and unable to maintain H3K79 methylation.

Thus, we theorize that nuclear export of AF10 may contribute to leukemogenesis by physically displacing DOT1L, resulting in global H3K79 hypomethylation. To test this hypothesis, we analyzed the epigenetic profile of cells expressing CALM-AF10 or CALM(mutNES1+2)-AF10, a mutant in which the hydrophobic residues of the NES are changed to alanines (**Figure 10**). Murine embryonic fibroblasts (MEFs) were retrovirally transduced with empty vector, CALM-AF10 or CALM(NESmut1+2)-AF10, and protein expression was verified by western blot (**Figure 20A**). CALM-AF10 localizes primarily to the cytoplasm of MEFs. However, it should be noted that some punctate nuclear staining is detectable in CALM-AF10-expressing cells, likely reflecting the fact that CALM-AF10 shuttles between the nucleus and the cytoplasm. As expected, CALM(NESmut1+2)-AF10 localizes exclusively to the nuclei of MEFs (**Figure 20B**).

We analyzed levels of di-methylated H3K79 in MEFs expressing cytoplasmic or nuclear CALM-AF10. In agreement with published results (Lin et al., 2009; Stoddart et al., 2012), stable expression of CALM-AF10 resulted in a 70% decrease in di-methylated H3K79 compared to the vector control (**Figure 20C**, middle lane; quantified in lower graph). However, expression of CALM(mutNES1+2)-AF10 did not affect H3K79 methylation (**Figure 20C**, right lane; quantified in lower graph). These results suggest that nuclear export of CALM-AF10 may be required for its ability to interfere with

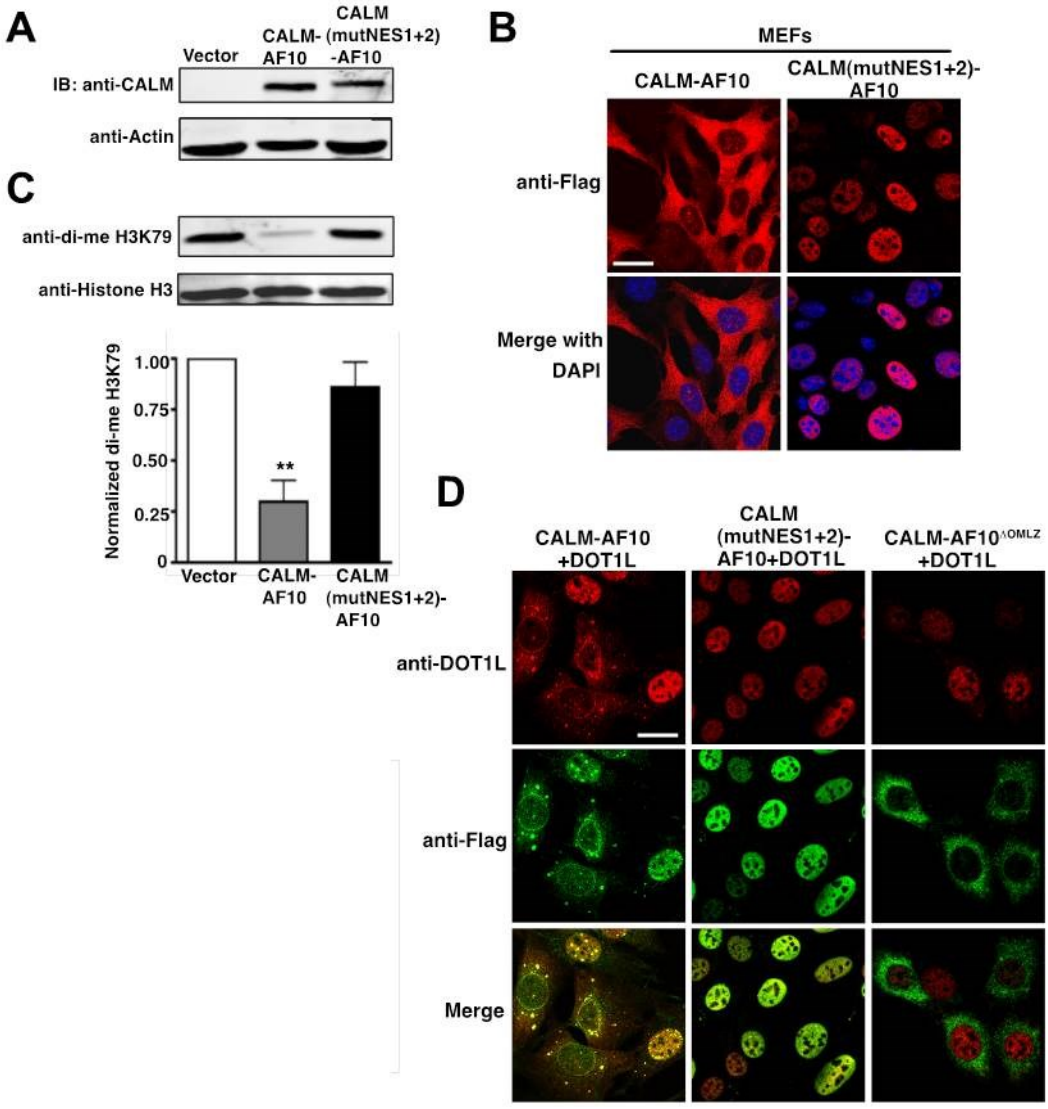

Figure 20: The CALM NES is necessary for H3K79 hypomethylation, possibly by cytoplasmic mislocalization of DOT1L

(**A-B**) Western blot (**A**) and confocal IF (**B**) of MEFs stably infected with empty vector, CALM-AF10, and CALM(mutNES1+2)-AF10. Data are representative of one set of MEFs (out of 3) stably infected with the respective constructs. (**C**) Representative western blot of di-methylated H3K79 and Histone H3 levels in stably infected MEFs. Quantification data (lower panel) represent the mean ± SEM of di-methylated H3K79 values normalized to Actin from separately generated MEF lines for each construct (n=3). Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test using empty vector-transduced MEFs as the control; **P<0.01 (**D**) Confocal IF of MEFs co-expressing DOT1L (red) and Flag-tagged CALM-AF10 (green, left), CALM(NESmut1+2)-AF10 (green, middle), or CALM-AF10ΔOMLZ (green, right). Scale bar represents 20 μm.

DOT1L activity.

Exclusion of DOT1L from the nucleus could explain the global loss of H3K79 methylation observed in CALM-AF10-expressing cells. To test whether CALM-AF10 directly mislocalizes DOT1L from the nucleus, DOT1L was co-expressed with CALM-AF10 or CALM(NESmut1+2)-AF10 in MEFs. As shown in **Figure 20D**, CALM-AF10 and DOT1L co-localize. The distribution is variable, with 56% of cells expressing CALM-AF10 and DOT1L in the cytoplasm and 44% of cells displaying predominantly nuclear distribution of CALM-AF10 and DOT1L (**Figure 20D**, left panels). In contrast, CALM(NESmut1+2)-AF10 and DOT1L co-localize exclusively in the nucleus (100% of the cells) (**Figure 20D**, middle panels). As previously reported (Okada et al., 2006), DOT1L remains nuclear in the presence of a CALM-AF10 mutant missing the OM-LZ domain (AF10 aa 710-783; CALM-AF10$^{\Delta OMLZ}$) (**Figure 20D**, right panels). These results emphasize that full-length CALM-AF10 and DOT1L co-localize, and in the presence of CALM-AF10, DOT1L becomes cytoplasmic in approximately half of the cells. Therefore, export of AF10 out of the nucleus may displace a portion of DOT1L from the nucleus, resulting in its inability to methylate histones.

3.2.3 The CALM NES is necessary for H3K79 hypermethylation on the *Hoxa* locus

In **Figure 20**, we determined that CALM-AF10 displaces a portion of DOT1L from the nucleus, which correlates with its loss of function. However, while global H3K79 methylation is reduced, it has been reported that the *Hoxa5* promoter is locally

hypermethylated in CALM-AF10 cells (Okada et al., 2006). Indeed, we observed enriched H3K79 di-methylation across the *Hoxa* locus in both CALM-AF10 and NES^{CALM}-AF10 leukemia cells (**Figure 16**). These findings suggest that DOT1L is aberrantly targeted to *Hoxa* loci by CALM-AF10, and fusion of the NES to AF10 is sufficient for these epigenetic changes on the cluster. Therefore, we hypothesized that in addition to mediating a global loss of methylation, the CALM-derived NES is also necessary for local H3K79 methylation on the *Hoxa* locus.

We assessed H3K79 methylation levels at the *Hoxa* locus in MEFs expressing CALM-AF10 or CALM(mutNES1+2)-AF10 by chromatin immunoprecipitation (ChIP). As shown in **Figure 21A**, H3K79 di-methylation is enriched at the promoters of the *Hoxa* genes in CALM-AF10-expressing cells. In contrast, CALM(mutNES1+2)-AF10 cells exhibit a H3K79 methylation pattern similar to that of empty vector expressing cells. Therefore, aberrant recruitment of DOT1L to the *Hoxa* locus is dependent on the CALM NES motif.

The observation that H3K79 is aberrantly methylated in an NES-dependent manner led us to examine whether Leptomycin B could prevent DOT1L targeting to the *Hoxa* locus in CALM-AF10 cells. Vector or CALM-AF10 expressing MEFs were incubated with 1 nM LMB for 24 hr, and di-methyl H3K79 ChIP was performed. LMB incubation reduced H3K79 methylation on the *Hoxa* promoter regions in CALM-AF10 cells by about 40% (**Figure 21B**). However, LMB did not significantly alter H3K79 methylation in vector

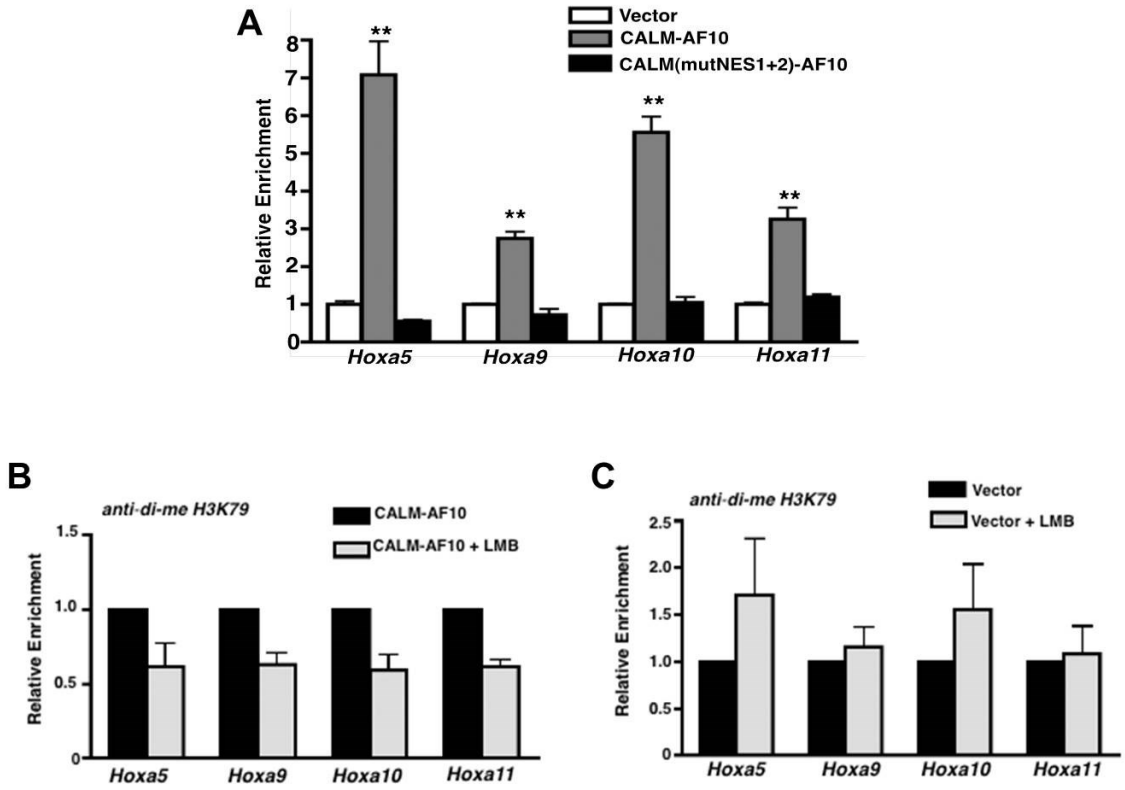

Figure 21: CALM-AF10-mediated H3K79 hypermethylation on the *Hoxa* locus is dependent on CRM1 interaction with the CALM NES

(**A-C**) ChIP analysis of di-methylated H3K79 in the promoter regions of the *Hoxa* cluster genes. (**A**) White bars represent empty vector, grey bars are CALM-AF10 and black bars are CALM(mutNES1+2)-AF10. *Hoxa* amplification was measured by Real Time PCR as a percent of input then normalized to the vector control. Results are shown as mean ± SEM from separately generated MEF lines for each construct (n=3). Statistical analyses were performed using one-way ANOVA for each *Hoxa* gene followed by Dunnett's multiple comparison test. Only CALM-AF10 was found to be significantly different from the vector control; **P<0.01. (**B**) CALM-AF10- (**C**) or Vector-expressing cells untreated (black bars) or treated with 1 nM LMB for 24 hr (light grey bars) followed by di-me H3K79 ChIP. Results are shown as percent of input normalized to the untreated conditions (mean ± SEM compiled from 3 independent experiments). A statistically significant reduction (Student's t-test; P<0.05) in di-methyl H3K79 ChIP was observed at *Hoxa9* and *Hoxa11* in LMB-treated CALM-AF10 cells.

expressing cells (**Figure 21C**). These results suggest that a CRM1-NES interaction is critical for DOT1L targeting and/or activity at the *Hoxa* locus in CALM-AF10 cells.

3.2.4 CALM-AF10 is directly targeted to *Hoxa* chromatin in a CRM1-dependent manner

We determined that H3K79 hypermethylation of *Hoxa* promoters is dependent on the CALM-derived NES (**Figure 21A**) via an interaction with CRM1 (**Figure 21B**). Because the NES interacts with CRM1 to mediate nuclear export of CALM-AF10, the effects on the *Hoxa* loci may be indirect, possibly by mislocalizing important transcriptional/epigenetic regulators to the cytoplasm. However, Okada et al. (2006) showed by ChIP that CALM-AF10 binds *Hoxa5* DNA. Therefore, these results suggest that DOT1L may be directly recruited to *Hoxa* chromatin by CALM-AF10, possibly in an NES-dependent manner.

To assess whether CALM-AF10 physically associates with the *Hoxa* cluster, we performed ChIP using an anti-Flag antibody and amplified regions of the *Hoxa* locus by Real Time PCR (**Figure 22A**). As shown in **Figure 22B**, CALM-AF10 binds to the *Hoxa* locus, specifically at *Hoxa9* and *Hoxa10*. However, we did not observe enrichment of CALM-AF10 within the *Hoxa5* coding region or promoter. This is in contrast to the results of Okada et al. (2006), who reported that CALM-AF10 binds within the *Hoxa5* coding region. Intriguingly, the pattern of CALM-AF10 association with *Hoxa9* is similar to that reported for MLL-AF10 (Okada et al., 2005).

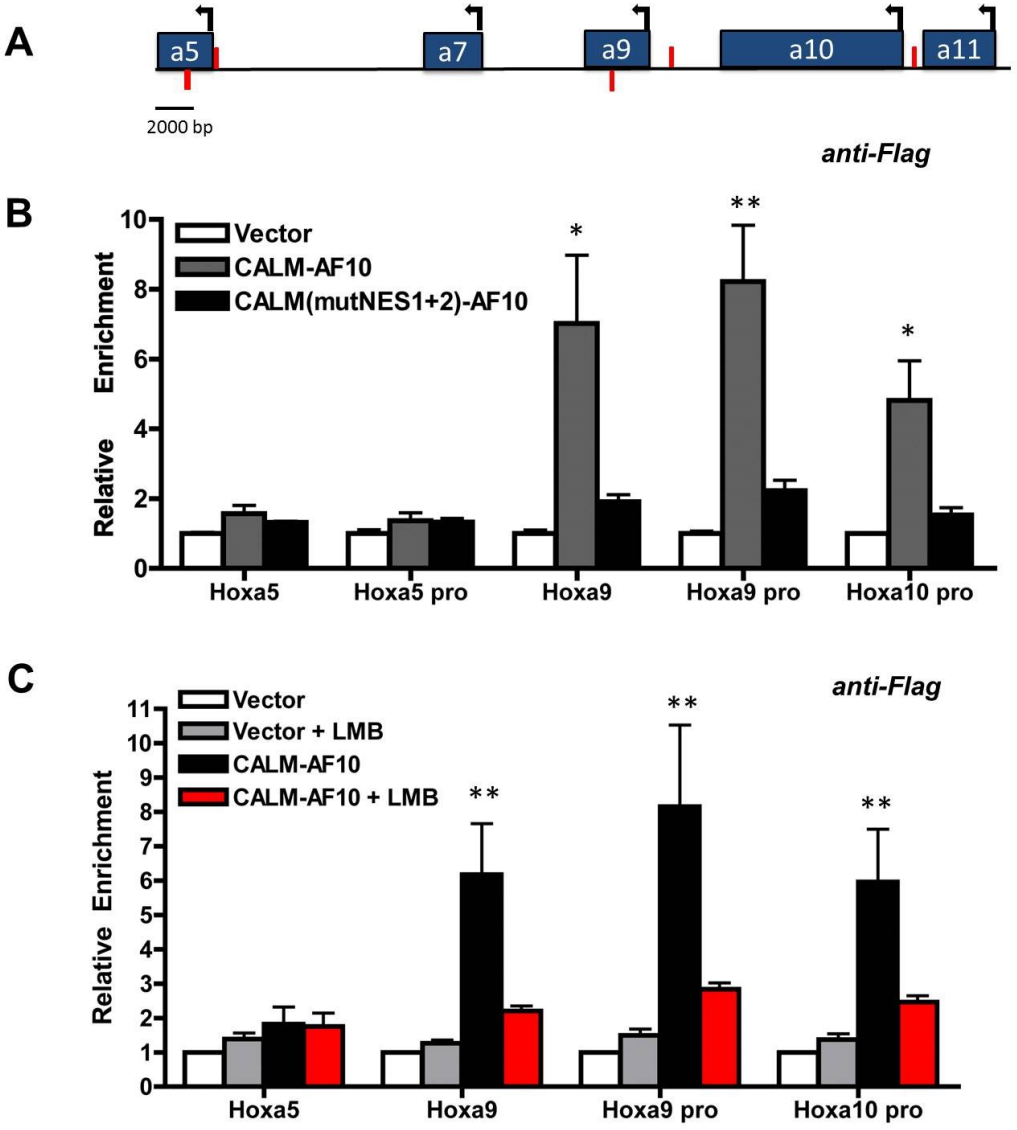

Figure 22: CALM-AF10 association with *Hoxa* loci is dependent on CRM1 interaction

(**A**) Schematic of the *Hoxa* locus. Blue boxes represent each *Hoxa* gene, and black arrows indicate the transcription start site (TSS). Red bars depict the region amplified by PCR; amplicons upstream of the TSS are referred to as promoters (pro). (**B**) ChIP was performed using anti-Flag antibodies in MEFs expressing Vector, CALM-AF10, or CALM(mutNES1+2)-AF10. *Hoxa* amplification was measured by Real Time PCR as a percent of input then normalized to the vector control. Results are shown as mean ± SEM from separately generated MEF lines for each construct (n=3). (**C**) Vector or CALM-AF10-expressing cells were treated with 0.7 nM LMB for 2.5 hr, and anti-Flag ChIP was performed. Results are shown as mean ± SEM compiled from 4 independent experiments. Statistical analyses were performed using one-way ANOVA for each *Hoxa* gene followed by Dunnett's multiple comparison test. Only CALM-AF10 is significantly different from the vector control; *P<0.05, **P<0.01.

Next, we sought to elucidate whether CALM(mutNES1+2)-AF10 associates with chromatin. Because, the NES point mutant is exclusively nuclear, we would predict that it would be in closer proximity to bind DNA. However, we found that unlike CALM-AF10, CALM(mutNES1+2)-AF10 is not enriched at the *Hoxa* locus by Flag ChIP (**Figure 22B**).

Because the NES point mutant does not physically associate with the *Hoxa* locus, we hypothesized that the CALM-CRM1 interaction is necessary for targeting CALM-AF10 to chromatin. To test this hypothesis, we treated CALM-AF10 expressing cells with 0.7 nM LMB for 2.5 hr and performed Flag ChIP. Strikingly, this short treatment with LMB abolished the ability of CALM-AF10 to ChIP to the *Hoxa* locus (**Figure 22C**). These results strongly implicate that NES-mediated interaction with CRM1 is necessary for targeting CALM-AF10 and DOT1L to *Hoxa* chromatin.

3.2.5 The CALM-derived NES and CRM1 are necessary for transcriptional upregulation of the *Hoxa* cluster

We observed that CALM-AF10 and di-methylated H3K79 are enriched at the *Hoxa* locus in an NES-dependent manner. H3K79 di-methylation is a mark of active transcription, and the transforming potential of CALM-AF10 has been linked to transcriptional upregulation of *Hoxa* cluster genes (Caudell and Aplan, 2008; Caudell et al., 2007; Dik et al., 2005). As shown in Chapter 2, CALM-AF10 and NES^{CALM}-AF10 leukemia cells have elevated H3K79 methylation and up-regulated *Hoxa* gene expression (**Figures 15-16**). Therefore, we assessed whether CRM1-mediated targeting of CALM-AF10 and DOT1L results in elevated expression of the *Hoxa* cluster genes.

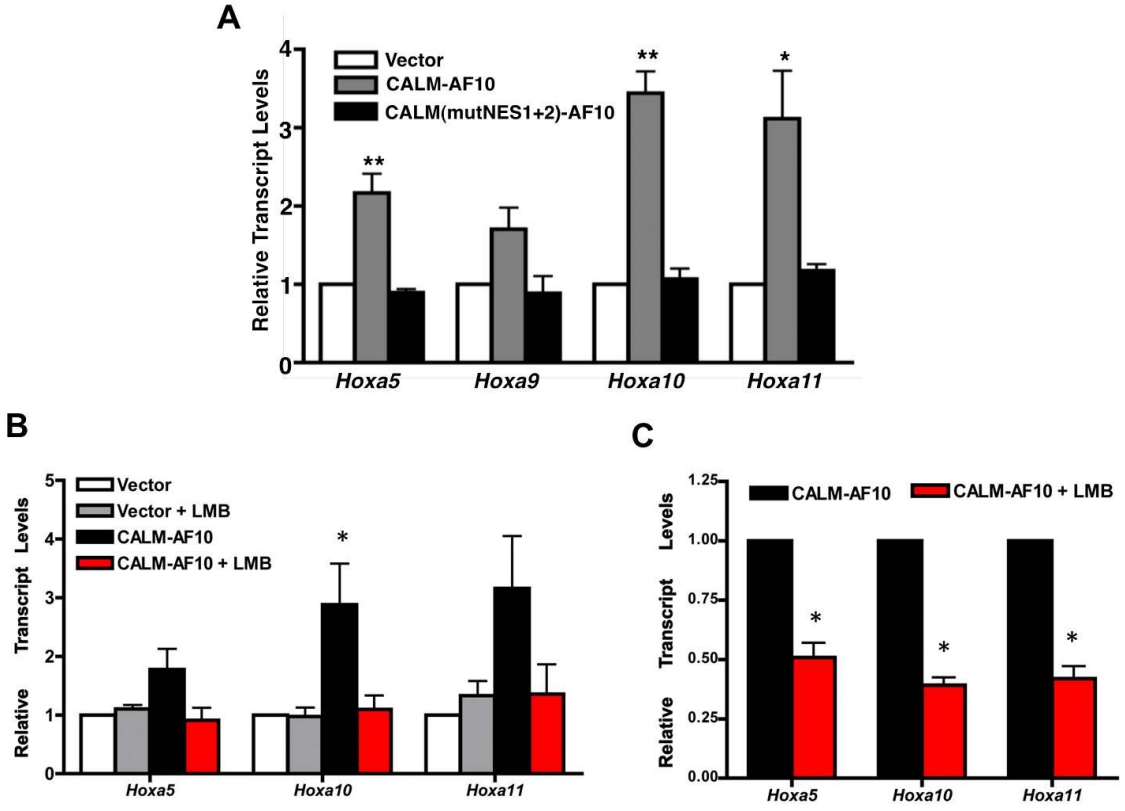

Figure 23: The CALM NES is necessary for elevated *Hoxa* cluster expression and short treatment with LMB prevents CALM-AF10-mediated *Hoxa* expression

(**A**) MEFs were stably infected with empty vector, CALM-AF10, or CALM(mutNES1+2)-AF10. *Hoxa* transcript levels were measured by Real Time RT-PCR and normalized to housekeeping genes GAPDH and β2M and then to empty vector-infected MEFs by the ΔΔCt method. Results are shown as mean ± SEM from separately generated MEF lines for each construct (n=3) (**B**) Vector or CALM-AF10-expressing cells were treated with 0.7 nM LMB for 2.5 hr, and Real Time RT-PCR was performed. *Hoxa* transcript levels were normalized to *GAPDH* and then to the vector control. (**C**) To represent the percent change in *Hoxa* expression in the CALM-AF10 cells treated with LMB, *Hoxa* transcript levels were normalized to GAPDH and then to the untreated control. For panels B-C, results are shown as mean ± SEM from 3 separate experiments. For panels A-B, statistical analyses were performed using one-way ANOVA for each *Hoxa* gene followed by Dunnett's multiple comparison test. Only CALM-AF10 was found to be significantly different from the vector control; **P<0.01, *P<0.05. For panel C, a statistically significant reduction (Student's t-test; *P<0.05) in *Hoxa* transcript levels was observed in LMB-treated CALM-AF10 cells.

We performed Real Time RT-PCR in MEFs stably expressing CALM-AF10 or CALM(mutNES1+2)-AF10. As shown in **Figure 23A**, expression of CALM-AF10 correlates with elevated transcript levels of *Hoxa5* (2-fold), *Hoxa9* (1.7-fold), *Hoxa10* (3.5-fold), and *Hoxa11* (3-fold) compared to empty vector. In contrast, expression of CALM(mutNES1+2)-AF10 did not significantly change *Hoxa* expression levels (**Figure 23A**). Together, these data support the importance of the CALM NES in targeting CALM-AF10 to *Hoxa* chromatin. Because short incubation with LMB prevents CALM-AF10 enrichment at the *Hoxa* locus, we assessed the downstream effects on *Hoxa* gene expression. Vector or CALM-AF10 expressing MEFs were incubated with 0.7 nM LMB for 2.5 hr, RNA was extracted and Real Time RT-PCR was performed. As shown in **Figure 23B**, incubation with LMB specifically reduced *Hoxa* transcript levels in CALM-AF10 cells. A 50-60% reduction was observed after 2.5 hr LMB treatment (**Figure 23C**). While H3K79 di-methylation is reduced in CALM-AF10 cells after 24 hr LMB treatment (**Figure 21B**), both CALM-AF10 binding and *Hoxa* transcript levels are lost after only 2.5 hr. These results suggest that CALM-AF10 has a direct effect on *Hoxa* expression that is separate from its role in mediating H3K79 methylation. The rapid response to LMB treatment also suggests that these effects are not due to cytotoxic or off-target effects of blocking all CRM1-mediated nuclear export.

In contrast to retrovirally infected MEFs, CALM-AF10 is expressed at relatively low levels in leukemia cells. Therefore, attempts to ChIP CALM-AF10 in this

physiologically relevant setting have been unsuccessful (data not shown). To verify the observations made in MEFs in a more relevant cell type, murine *CALM-AF10* leukemia cells were treated with 0.7 nM LMB for 2.5 hr, and *Hoxa* transcript levels were assessed. As shown in **Figure 24A**, short incubation with LMB resulted in a 60% loss of *Hoxa* transcript levels in *CALM-AF10* cells, similar to that observed in stably infected MEFs. In comparison, *Hoxa* transcript levels were unchanged in *Hoxa9*/*Meis1* cells after 2.5 hr LMB treatment (**Figure 24B**). Therefore, CALM-AF10-mediated upregulation of the *Hoxa* cluster genes is dependent on CRM1 interaction in leukemic cells.

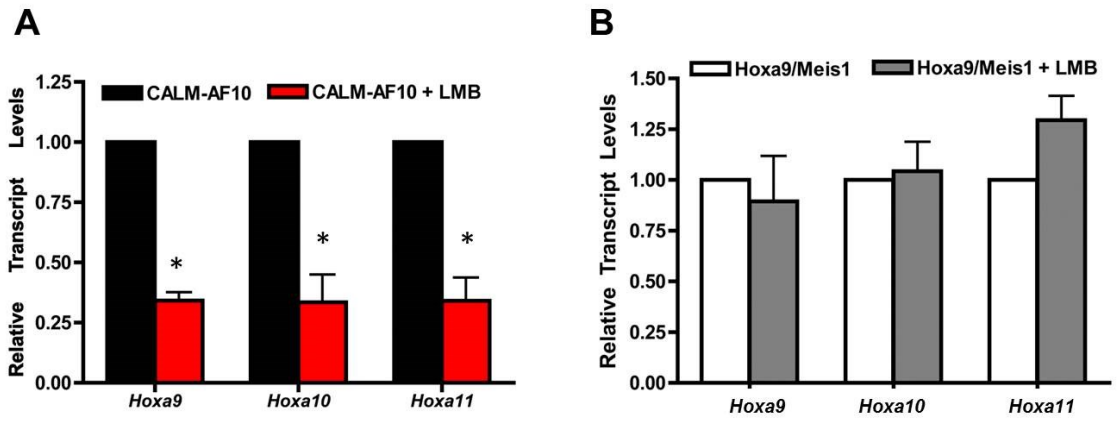

Figure 24: Short treatment with LMB results in loss of *Hoxa* expression in *CALM-AF10* murine leukemia cells

(**A-B**) Cell lines were generated by culturing bone marrow blasts from mice with *CALM-AF10* (**A**) or *Hoxa9*/*Meis1* (**B**) leukemias. Cells were treated with 0.7 nM LMB for 2.5 hr, and Real Time RT-PCR was performed. *Hoxa* transcript levels were normalized to *GAPDH* and then to the untreated control. Results are shown as mean ± SEM from 3 separate experiments. Statistical analysis was performed by Student's t-test. *CALM-AF10* cells treated with LMB (panel A, red bars) had significantly reduced *Hoxa* transcript levels than the untreated cells *P<0.05. LMB treatment did not significantly affect *Hoxa* transcript levels in *Hoxa9*/*Meis1*-derived leukemias.

It is generally accepted that CALM-AF10 functions to recruit DOT1L to the *Hoxa* locus, resulting in H3K79 hypermethylation and subsequent *Hoxa* upregulation. However, short incubations with LMB prevent CALM-AF10 from binding chromatin and lead to a rapid reduction in *Hoxa* transcript levels. These effects occur prior to decreases in H3K79 methylation, suggesting that CALM-AF10 is directly involved in either transcriptional control or stability of *Hoxa* transcripts.

To assess whether CALM-AF10 affects transcription of the *Hoxa* cluster genes, we compared cells treated with LMB or Actinomycin D (ActD). ActD binds DNA and prevents RNA Polymerase-mediated transcriptional elongation. Therefore, following treatment with ActD, the levels of *Hoxa* transcripts should be dependent on their stability rather than active transcription. If *Hoxa* transcript levels are reduced in a similar manner with ActD or LMB then we would assume that LMB directly affects transcription by blocking CALM-AF10. As shown in **Figure 25**, incubation with ActD (5 μg/ml) results in loss of *Hoxa* and *GAPDH* transcript levels over time. Likewise, treatment with LMB (0.7 nM) results in reduction of *Hoxa* transcript levels with kinetics similar to ActD treatment. Importantly, *GAPDH* transcript levels are not decreased in LMB treated cells, emphasizing that LMB does not block all active transcription in CALM-AF10 cells. Therefore, we conclude that CALM-AF10 transcriptionally activates the *Hoxa* locus. Treatment with LMB prevents CALM-AF10 binding to chromatin, resulting in loss of

Hoxa transcription. Additional experiments are necessary to elucidate the specific mechanism by which this occurs.

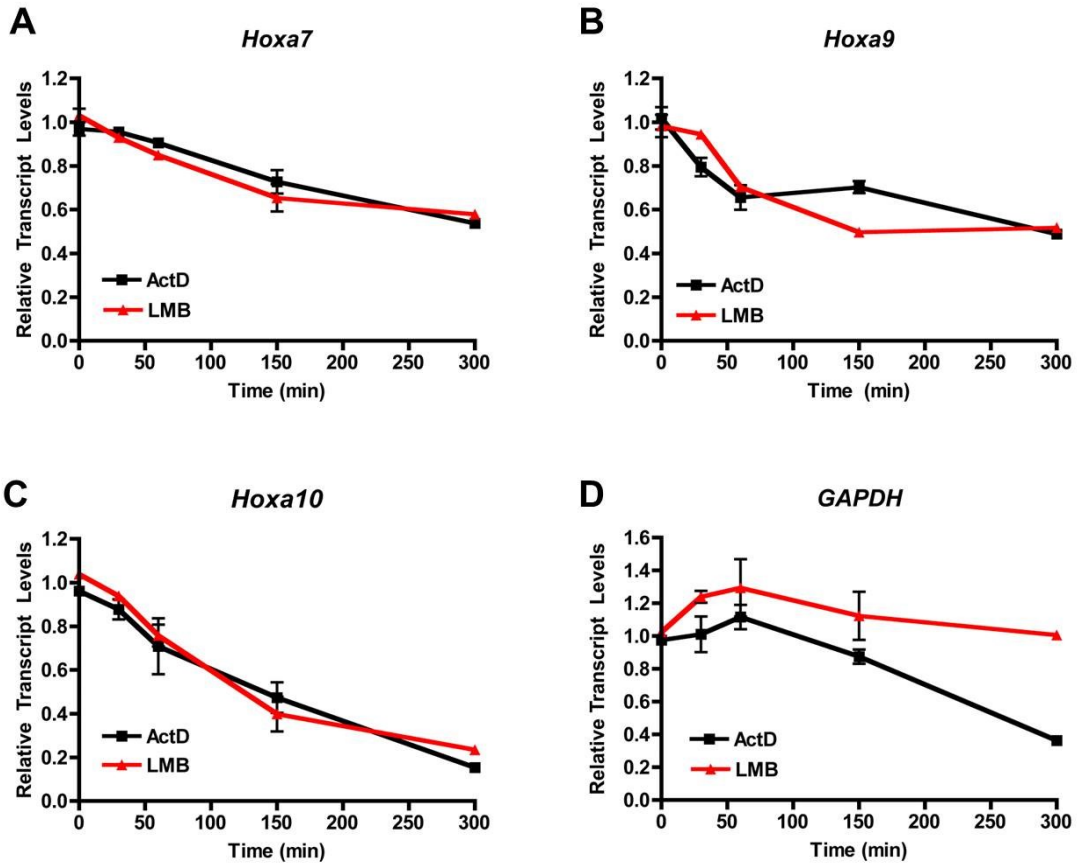

Figure 25: LMB and Actinomycin D inhibit *Hoxa* expression with similar kinetics in *CALM-AF10* leukemia cells

(**A-D**) *CALM-AF10* murine leukemia cell lines were treated with 5 μg/ml Actinomycin (ActD) or 0.7 nM LMB for 30, 60, 150, or 300 min, and Real Time RT-PCR was performed. *Hoxa7* (A), *Hoxa9* (B), *Hoxa10* (C), or *GAPDH* (D) transcript levels were normalized to the untreated condition. Results are shown as mean ± SEM from 2 separate experiments. While treatment with LMB or ActD results in similar loss of *Hoxa* transcript levels over time, *GAPDH* levels were unchanged with LMB treatment in *CALM-AF10* cells.

3.3 Discussion

Structure-function analysis of CALM-AF10 uncovered a requirement for a CRM1-dependent nuclear export signal. This finding was surprising because it is the first demonstration that aberrant fusion of an NES to a nuclear protein results in leukemic transformation. In this Chapter, we extended our observations of the key structural components of *CALM-AF10* to elucidate the molecular mechanisms by which the CALM-derived NES imparts transformation potential to AF10. Specifically, we inquired whether steady-state cytoplasmic localization or nucleocytoplasmic shuttling of AF10 are necessary for leukemogenesis. While we found that nuclear export of AF10 results in displacement of DOT1L to the cytoplasm, we also determined that the NES-CRM1 interaction is critical for targeting DOT1L to *Hoxa* loci. CRM1-dependent recruitment of CALM-AF10 (and thus DOT1L) to the *Hoxa* cluster genes is necessary for their transcription. Together, these results not only provide insights into the mechanism by which CALM-AF10 is leukemogenic, but also suggest a novel role for CRM1 in gene regulation.

We first asked whether ectopic cytoplasmic localization of the AF10 carboxy-terminus is sufficient for transformation. This construct is cytoplasmic, and its localization does not change in the presence of LMB. However, cytoAF10 is unable to transform murine HPs in *in vitro* transformation assays. Although we cannot rule out the possibility that cytoAF10 is not properly expressed, these results suggest that fusion of a

CRM1-dependent NES to AF10 is critical for transformation. Indeed, additional mechanistic studies of the NES support the notion that cytoplasmic retention of AF10 is not, on its own, oncogenic.

To explore the mechanistic requirement for the CALM-derived NES, we compared MEFs ectopically expressing CALM-AF10 or CALM(mutNES1+2)-AF10. Immortalized MEFs were used because they are relatively easy to infect and are not transformed by CALM-AF10. Therefore, we could assess the direct downstream effects of CALM-AF10 that are not a side effect of transformation.

Here, we demonstrate that nuclear export of AF10 correlates with a perturbed epigenetic state. *CALM-AF10* leukemias are characterized by a global reduction in H3K79 methylation, and this phenotype may contribute to leukemogenesis by increasing chromosomal instability (Lin et al., 2009). We determined that while expression of CALM-AF10 results in a dramatic loss of H3K79 methylation, cells that express CALM(mutNES1+2)-AF10 do not display altered H3K79 methylation. It has previously been proposed that CALM-AF10 acts in a dominant negative manner on endogenous DOT1L to cause global H3K79 hypomethylation (Lin et al., 2009). Because the OM-LZ domain of AF10 is necessary and sufficient for CALM-AF10-mediated transformation, we theorized that CALM-AF10 might alter the subcellular localization of the OM-LZ binding partner DOT1L. Displacement of DOT1L from the nucleus could then result in a global loss of H3K79 methylation. Indeed, we observe that while DOT1L is nuclear in the

presence of CALM(mutNES1+2)-AF10 or CALM-AF10ΔOMLZ, CALM-AF10 and DOT1L co-localize in both the cytoplasm and the nucleus. Therefore, cytoplasmic CALM-AF10 correlates with loss of DOT1L function, which may directly contribute to global alterations in gene expression and an unstable genome.

It is possible that nuclear export of CALM-AF10 also results in the displacement of other OM-LZ binding partners. For example, CALM-AF10 has been shown to alter the localization and function of IKAROS, but the consequences of this mislocalization have not been elucidated (Greif and Bohlander, 2011; Greif et al., 2008). In addition, GAS41 is another OM-LZ binding protein that functions as a component of the SWI/SNF complex, and its mislocalization could potentially result in transcriptional deregulation (Debernardi et al., 2002). Further investigations are necessary to determine the effects of nuclear export of CALM-AF10 on endogenous AF10 and its known OM-LZ binding partners.

In addition to global loss of H3K79 methylation, CALM-AF10 leukemia cells are locally H3K79 hypermethylated at the *Hoxa* loci. DOT1L-dependent H3K79 methylation is correlated with up-regulation of *Hoxa* expression in both *MLL*-rearranged and *CALM-AF10* leukemias (Okada et al., 2005; Okada et al., 2006). Likewise, both CALM-AF10 and MLL-AF10 have been found to directly interact with *Hoxa* chromatin. Therefore, CALM-AF10 is thought to target DOT1L to *Hoxa* genes, resulting in elevated H3K79 methylation and increased transcription. Because upregulation of *Hoxa* genes is a critical driver of

leukemogenesis, we asked whether the CALM NES is necessary to mediate these effects. Surprisingly, we found that both H3K79 hypermethylation and CALM-AF10 targeting to *Hoxa* chromatin are dependent on the ability of CALM to interact with CRM1. It is possible that CALM-AF10 directly binds chromatin as it undergoes nucleocytoplasmic shuttling. Likewise, the nuclear pore has been characterized as a site of active gene transcription in eukaryotes (Brown and Silver, 2007). Therefore, it is conceivable that the CALM-derived NES targets CALM-AF10 to the nuclear periphery (via CRM1), where *Hoxa* locus-specific gene activation may occur. On the other hand, numerous nucleoporins (Nups) have been found to dissociate from the NPC at the nuclear periphery, bind chromatin, and activate gene transcription within the nucleoplasm (Arib and Akhtar, 2011; Capelson et al., 2010b; Kalverda et al., 2010; Liang et al., 2013). Therefore, it is conceivable that CRM1 may independently or via an interaction with Nups regulate gene expression. However, a role of CRM1 in transcriptional regulation has not been described in mammalian cells to date.

Intriguingly, another leukemic fusion protein, SET-NUP214, was found to bind both CRM1 and DOT1L (Van Vlierberghe et al., 2008). *SET-NUP214* leukemic cells also have up-regulated *Hoxa* gene expression and local H3K79 hypermethylation, supporting a potential oncogenic mechanism involving DOT1L and CRM1 (Van Vlierberghe et al., 2008). In addition, new AF10 fusion partners, *NAP1L1, HNRNPH1, and DDX3X* have recently been identified in T-ALLs (Brandimarte et al., 2012; Zhang et al., 2012). Similar to

CALM, NAP1L1 and DDX3X contain characterized CRM1 interaction domains, and hnRNP H1 has a putative NES (Miyaji-Yamaguchi et al., 2003; Sharma and Bhattacharya, 2010). Therefore, CRM1 interaction may be a recurrent feature of leukemic fusion proteins, emphasizing its potential role in mediating leukemogenesis. Further mechanistic studies aimed at understanding the role played by CRM1 interaction in leukemogenesis are needed.

Finally, we observed that elevated transcription of the *Hoxa* cluster genes is a direct result of CALM-AF10 interaction with CRM1. Short treatment with LMB reduces *Hoxa* transcript levels with kinetics similar to those seen with Actinomycin D-mediated interference with RNA polymerase. Prior to these studies, CALM-AF10 was thought to up-regulate *Hoxa* expression via DOT1L-mediated H3K79 hypermethylation. Methylated H3K79 correlates with active transcription, however it has been unclear if it is necessary for transcription to occur. Here, we found that treatment with LMB for less than 5 hr reduces transcription of *Hoxa* genes prior to loss of H3K79 methylation. Therefore, CALM-AF10 is directly involved in transcription of *Hoxa* genes, which is independent of DOT1L-mediated H3K79 methylation. At this point, it is unclear how CALM-AF10 transcriptionally activates the *Hoxa* cluster genes. These effects may still be dependent on DOT1L and its role in transcriptional elongation complexes with or without AF10 (Lin et al., 2010; Smith et al., 2011a). Alternatively, other transcriptional regulators may be recruited to the *Hoxa* locus either through AF10 or CALM.

In summary, the findings presented in this Chapter elucidate mechanisms by which a CALM-derived NES confers transformation potential to AF10. Nuclear export of AF10 mislocalizes a portion of DOT1L to the cytoplasm, which correlates with a global loss of H3K79 methylation. In contrast, CALM-AF10 binding, H3K79 hypermethylation, and transcriptional upregulation at the *Hoxa* locus are mediated by NES-CRM1 interaction within the nucleus. Further studies are warranted to elucidate the endogenous and leukemogenic role of CRM1 at *Hoxa* chromatin. Finally, because of the drastic effects observed following treatment with LMB, we hypothesize that CRM1 inhibitors may represent a novel therapeutic approach for patients with *CALM-AF10* leukemias.

4. Conclusions and Perspectives

4.1 The CALM NES and AF10 OM-LZ motif are the functional domains for CALM-AF10-mediated leukemogenesis

Leukemic chromosomal translocations often involve juxtapositioning of normally separate genes, resulting in formation of hybrid fusion proteins with altered function. Translocations involving the *CALM* and *AF10* genes are recurrent in aggressive human leukemias. The oncogenic effects of the CALM-AF10 fusion have been validated using transgenic and retroviral transplantation mouse models. Although ectopic expression of either CALM or AF10 alone is not oncogenic, abnormal expression of the CALM-AF10 fusion imparts leukemic properties to cells. Therefore, the oncogenic potential of CALM-AF10 is due to the combined function of two regions, one within CALM and one within AF10. Elucidation of these domains is critical to understanding the mechanism(s) by which CALM-AF10 causes leukemia.

In Chapter 1, we performed structure-function analysis to determine the critical domain within CALM for *CALM-AF10*-mediated leukemogenesis. We found that a CRM1-dependent nuclear export signal (NES) is both necessary and sufficient for transformation. AF10 is a nuclear protein that does not contain a conserved NES, and ectopic fusion of this domain to AF10 results in gain of transformation potential. To verify that the sole contribution of CALM to the fusion is an NES, we fused conserved

NES motifs from heterologous proteins to AF10 and assessed their transformation ability. Indeed, fusion of the NES motifs from ABL1, PKIA, Rev, or APC to AF10 is sufficient for immortalization. However, fusion of the conserved NES from the MEK1protein to AF10 did not transform murine HPs. This result was surprising because NES^{MEK1}-AF10 is a cytoplasmic protein that undergoes nucleocytoplasmic shuttling with similar kinetics as the other NES fusions, and therefore should contain all of the necessary components for transformation. It is possible that through a separate function, the MEK1 NES is inhibitory or does not allow for proper folding of AF10. Alternatively, altered structure or sequence of the MEK1 NES may prevent recruitment of a binding protein separate from CRM1. In the future, it would be informative to perform comparative studies of the CALM and MEK1 NES to provide further insight into the critical components of this motif.

Because the MEK1 NES fused to AF10 did not transform murine HPs, it would also be important to verify the necessity of CRM1 recruitment to AF10. If the NES motif functions solely to recruit CRM1, then it can be hypothesized that direct fusion of CRM1 to AF10 may be oncogenic on its own. This finding would eliminate the possibility that the NES has another structural or functional role besides binding CRM1. Likewise, although we found that NES^{CALM}-AF10 is sufficient for leukemogenesis *in vivo*, these leukemias developed with a longer latency than CALM-AF10 or CALM(520-583)-AF10 leukemias. Structural or expression differences between NES^{CALM}-AF10 and CALM(520-

583)-AF10 could contribute to this phenotype, but it is also possible that a region outside of the NES (within aa 520-583) is necessary to generate a more potent leukemia. Therefore, rigorous assessment of whether ectopic CRM1 recruitment to AF10 is the sole contribution of CALM will be an area of further investigation.

It has previously been determined that the OM-LZ domain within AF10 is both necessary and sufficient for *CALM-AF10*-mediated leukemogenesis. Combined with our structure-function analysis of CALM, we conclude that the NES and OM-LZ domains are the critical oncogenic components of CALM-AF10. Indeed, fusion of the CALM NES to the carboxy-terminal domain of AF10 (spanning OM-LZ) is sufficient for transformation *in vitro*. As the OM-LZ domain has been found to bind various proteins, it is not precisely clear how this region contributes to leukemogenesis. DOT1L, the H3K79 histone methyltransferase, interacts with AF10 via the OM-LZ domain. Because H3K79 is aberrantly methylated in *CALM-AF10* and *MLL-AF10* leukemias (Lin et al., 2009), it is presumed that the DOT1L-AF10 interaction is critical. Interestingly, one group found that fusion of MLL to DOT1L is sufficient for transformation *in vitro* (Okada et al., 2005); however this finding could not be replicated by others (Yokoyama et al., 2010). Although it is likely that aberrant DOT1L activity is necessary for leukemogenesis, it is unclear whether the OM-LZ domain solely functions to recruit DOT1L. Other OM-LZ binding partners include IKAROS and GAS41, two proteins involved in global gene regulation

(Debernardi et al., 2002; Greif et al., 2008). Therefore, it will be interesting to study the potential role of IKAROS and GAS41 in these leukemias.

In summary, the CALM NES and AF10 OM-LZ domains are the critical structural components of the CALM-AF10 fusion. The CALM NES interacts with the CRM1 nuclear export receptor, and additional studies are necessary to determine whether this is the exclusive oncogenic function of the NES motif. The AF10 OM-LZ domain interacts with the DOT1L methyltransferase, correlating with altered H3K79 methylation. The precise function of the OM-LZ domain is unclear, and it remains to be elucidated whether other OM-LZ binding partners are perturbed in *CALM-AF10* leukemias.

4.2 H3K79 is abnormally methylated in CALM-AF10 cells

Methylation of Histone H3 on Lysine 79 is ubiquitously correlated with active transcription (Steger et al., 2008). As discussed, DOT1L is the only mammalian H3K79-specific methyltransferase, and a demethylase has not been discovered to date. Therefore, DOT1L mono-, di-, and tri-methylates H3K79, and loss of methylation is dependent on histone turnover. DOT1L has also been found in large complexes with transcriptional elongation factors. These studies have corroborated the link between H3K79 methylation and transcription; however the exact mechanism by which these are related remains unclear. Specifically, it is unknown whether H3K79 methylation precedes or follows active transcriptional elongation. It is also unclear whether DOT1L plays a role in

transcriptional elongation that is separate from its role in histone methylation. These aspects of basic molecular biology have been outside the scope of our studies but are critical for understanding how gene regulation is perturbed in *CALM-AF10* and *MLL* leukemias.

CALM-AF10 leukemic cells are characterized by aberrant H3K79 methylation. Genome-wide, H3K79 is hypomethylated, suggesting that DOT1L is unable to adequately maintain this epigenetic mark. H3K79 hypomethylation is a direct result of CALM-AF10 expression and is specifically mediated by the AF10 OM-LZ domain (Lin et al., 2009). Therefore, it has been proposed that CALM-AF10 displaces DOT1L from chromatin. Here, we found that in addition to the OM-LZ domain, H3K79 hypomethylation is also dependent on the CALM NES. Using co-immunofluorescence, we observed a fraction of DOT1L mislocalized to the cytoplasm in the presence of CALM-AF10. Therefore, it can be rationalized that nucleocytoplasmic shuttling of CALM-AF10 results in displacement of DOT1L, leading to its loss of function. As these studies were performed in MEFs, it will be important to confirm the localization of DOT1L in a *CALM-AF10* leukemic cell.

Although our studies may provide a rationale for why H3K79 methylation is lost, the consequences of this aberrant epigenetic modification remain unknown. It has been shown that H3K79 hypomethylation correlates with increased DNA damage and subsequent chromosomal instability (Lin et al., 2009). Therefore, it can be theorized that

H3K79 hypomethylation contributes to the generation of additional genetic mutations or "hits". As CALM-AF10 leukemias develop with an extended latency, it has been suggested that additional mutations are necessary for complete penetrance of disease (Caudell and Aplan, 2008). Therefore, loss of DOT1L-mediated H3K79 methylation may result in random genetic mutations that confer a growth or survival advantage. Because H3K79 methylation is coupled with gene transcription, global hypomethylation could also lead to reduced expression of a number of genes. Consequently, CALM-AF10 might promote leukemogenesis by indirectly interfering with multiple cellular pathways.

In contrast, we and others have identified H3K79 hypermethylation at the *HOXA* cluster genes, which corresponds with their transcriptional upregulation. The dichotomy between global loss and local gain of H3K79 methylation is intriguing. The most mysterious aspect of altered H3K79 methylation is how and why certain genes are targeted and others are not. Is specific enrichment of H3K79 methylation due to a DOT1L-mediated hierarchy of target genes; or does CALM-AF10 exclusively recruit DOT1L to its own targets? Alternatively, our ChIP data show that interaction with CRM1 is necessary for recruitment of CALM-AF10 to the *Hoxa* locus. Therefore, it is possible that CRM1 actively targets CALM-AF10 and thus DOT1L to certain regions of the genome. A broader assessment of the epigenetic profile of *CALM-AF10* cells may provide insights into the regulation of these histone marks. For example, di-me H3K79 ChIP-seq would theoretically enrich for hypermethylated regions of the genome. Likewise, CRM1

ChIP-seq may correlate with methylated H3K79 profiles, providing the first evidence of potential CRM1 gene targets.

To assess the dichotomy of H3K79 methylation further, it would be interesting to evaluate the temporal dynamics of these epigenetic changes. One could envision that global loss of H3K79 methylation might have profound effects on the nuclear organization of chromosomes. Does loss of methylated H3K79 in some areas of the genome allow for other areas to become exposed or achieve a more open conformation? One way to test this possibility would be to express CALM-AF10 in an inducible manner and assess the immediate downstream epigenetic effects. Does the *Hoxa* locus become hypermethylated before global hypomethylation occurs, or is there a lag time? Answers to these questions will provide novel insights into the basic mechanisms of epigenetic organization and how perturbations of these processes contribute to cancer.

4.3 CALM-AF10 mediates transcriptional upregulation of Hoxa genes

As previously discussed, local H3K79 hypermethylation at the *Hoxa* cluster correlates with transcriptional upregulation of *HOXA* genes in *CALM-AF10* leukemias. Here, using ChIP techniques, we determined that CALM-AF10 interacts with the *Hoxa* locus. While others found CALM-AF10 enrichment within the coding region of *Hoxa5* (Okada et al., 2006), our results suggest that CALM-AF10 binds upstream at *Hoxa9* and *Hoxa10*. Okada et al. did not assess this region of the locus; therefore it is possible that

they also might have found enrichment of CALM-AF10 in this region. Additionally, we used Real Time PCR to amplify the *Hoxa* locus, while Okada et al. performed less sensitive semi-quantitative techniques. Therefore, technical differences could also account for the differing results. Nevertheless, our data also support the notion that CALM-AF10 binds chromatin, which is important in light of our finding that CALM-AF10 undergoes nuclear export.

While CALM-AF10 physically interacts with the *Hoxa* locus, CALM(mutNES1+2)-AF10 does not. Additionally, short treatment with LMB prevents CALM-AF10 from binding *Hoxa* DNA. Therefore, the NES is critical for targeting CALM-AF10 to the *Hoxa* locus, and this likely occurs via interaction with CRM1. These results are surprising because CRM1 binding to a NES motif has not previously been implicated in gene regulation. In the future, it will be necessary to perform CRM1 ChIP to verify that it directly binds to the *Hoxa* locus. It will also be interesting to test whether CRM1 interaction at the *Hoxa* locus is sensitive to LMB. If CRM1 does not physically associate with DNA in the presence of LMB, then other NES binding factors may also be important.

While we determined that the CALM-derived NES is essential for targeting CALM-AF10 to the *Hoxa* locus, the mechanism by which this occurs remains unknown. One possible explanation may be that CALM-AF10 binds chromatin as it undergoes nucleocytoplasmic shuttling. Because CRM1 mediates nuclear export of CALM-AF10

through the NPC, it is conceivable that *Hoxa* locus-specific gene activation may occur at the nuclear periphery. Likewise, the nuclear pore complex has been characterized as a site of active gene transcription in eukaryotes (Brown and Silver, 2007). Therefore, fluorescence *in situ* hybridization experiments could be performed to assess the proximity of *Hoxa* DNA to the nuclear envelope in *CALM-AF10* cells.

It is also possible that CALM-AF10 targets *Hoxa* loci via CRM1 within the nucleoplasm. Although CRM1 is a nuclear export receptor, its localization is not restricted to the nuclear periphery. In fact, CRM1 has been shown to localize to unique nuclear foci with the FG-nucleoporin NUP98 (Griffis et al., 2002; Oka et al., 2010). These nuclear foci are lost upon addition of either LMB or ActD, suggesting that they are dependent on CRM1 and active transcription. Although the function of CRM1 nuclear foci is unknown, NUP98 is a mobile nucleoporin that binds chromatin and activates transcription (Capelson et al., 2010b; Griffis et al., 2002; Liang et al., 2013). Therefore, it can be theorized that CRM1 might be recruited to chromatin via interaction with NUP98. Even more intriguing is the fact that *NUP98* is a leukemic translocation partner for a number of other genes, and *NUP98* leukemias have up-regulated expression of the *Hoxa* cluster genes. Altogether, a potential link exists between CRM1, NUP98, and transcriptional activation within the nucleoplasm. Additional studies aimed at assessing the presence of NUP98 at the *Hoxa* cluster are necessary. Finally, if recruitment to the *Hoxa* locus is solely dependent on a CALM-CRM1 interaction, then one can speculate that

endogenous CALM might also be targeted to chromatin. Therefore, it could be interesting to assess whether endogenous CALM interacts with CRM1 at the *Hoxa* locus.

In addition to where and why CRM1 mediates interaction with *Hoxa* loci is the question of *how* CALM-AF10 subsequently activates transcription. We have identified a significant correlation between CALM-AF10 interaction and transcription of *Hoxa* genes; yet the mechanism(s) by which this occurs remain unclear. The simplest explanation is that CALM mediates targeting to the *Hoxa* locus (via CRM1) and AF10 recruits DOT1L to add an active me-H3K79 mark. However, we found that short treatment with LMB prevents *Hoxa* transcription, while H3K79 methylation is unchanged. Therefore, the presence of H3K79 methylation is not sufficient for transcription.

As previously discussed, DOT1L is a component of transcriptional elongation complexes. In addition to methylating H3K79 at the *Hoxa* loci, it is possible that DOT1L also recruits components of these transcriptional complexes. Therefore, DOT1L may contribute to transcriptional phenotypes that are separate from its direct epigenetic modifications. Conversely, it is possible that in addition to DOT1L, AF10 recruits another transcriptional modifier through its OM-LZ domain. As discussed, potential candidates include GAS41 and IKAROS, and studies aimed at understanding the importance of these OM-LZ binding partners in CALM-AF10-mediated *Hoxa* transcription are necessary. On the other hand, it is possible that CRM1 recruits other transcriptional activators either on its own or via association with Nups. Likewise, it has been suggested

that CALM has transcriptional activity in a region spanning the NES (Archangelo et al., 2006), which could contribute to *Hoxa* cluster transcription. In summary, CALM-AF10 directly affects transcription of *Hoxa* cluster genes, and understanding the basic mechanisms by which this occurs will be a topic of future investigation.

4.4 CRM1 recruitment may be a common feature of leukemogenic fusion proteins

In addition to *MLL* and *CALM*, elegant sequencing studies have led to the discovery of previously unidentified *AF10* fusion partners in patients with T-ALLs. These include *NAP1L1, HNRNPH1,* and *DDX3X* (Brandimarte et al., 2012; Zhang et al., 2012). Interestingly, NAP1L1 and DDX3X contain characterized CRM1 interaction domains, and hnRNP H1 has a putative NES (Miyaji-Yamaguchi et al., 2003; Sharma and Bhattacharya, 2010). Therefore, CRM1 interaction may be a recurrent feature of leukemogenic AF10 fusion partners. Although the molecular phenotypes have not been studied, it is likely that other *AF10* leukemias rely on similar mechanisms as *CALM-AF10* leukemias, including aberrant H3K79 methylation and elevated *HOXA* cluster expression.

The observation that AF10 is translocated to other proteins with CRM1 interaction domains raises the question of whether MLL-AF10 causes leukemia by similar molecular mechanisms. Indeed, based on transcriptional and epigenetic profiles, *CALM-AF10* and *MLL-AF10* leukemias are extremely similar. While we determined that the effects of CALM-AF10 on epigenetics and *Hoxa* transcription are dependent on a CRM1-specific

NES, it is unclear whether the same can be concluded for MLL-AF10. MLL is a nuclear H3K4 methyltransferase and does not contain a conserved CRM1 binding domain. Therefore, it is not likely that MLL directly binds or recruits CRM1. However, it can be speculated that MLL may interact with a factor downstream of CRM1. An intriguing candidate is the nucleoporin NUP98, which was recently found to assist in maintenance of H3K4 methylation (Light et al., 2013). Although a direct association between NUP98 and MLL has not been elucidated, it is interesting to hypothesize that they could interact via regulation of H3K4 methylation. As an FG-Nup, NUP98 directly interacts with CRM1 both at the nuclear pore and within the nucleoplasm. Therefore, CRM1 interaction may a common element of *CALM-AF10*, *MLL-AF10*, and even *NUP98* leukemias. In addition to characterizing a potential CRM1 interaction, it could also be informative to assess the effects of LMB treatment on *Hoxa* expression in *MLL-AF10* and *NUP98* leukemias. Identification of a mechanistic link that relies on CRM1 interaction may have therapeutic implications for patients with these leukemias.

4.5 Therapeutic implications for patients with CALM-AF10 leukemias

Aggressive hematopoietic malignancies harboring *CALM-AF10* translocations are seen in both pediatric and adult patients and are associated with poor prognoses. Improving the outcome of *CALM-AF10* leukemias depends on the development of targeted therapies with improved efficacy and reduced toxicity. Based on our discovery

that immortalization by *CALM-AF10* is dependent on a CALM-derived NES, we hypothesize that nuclear export inhibitors represent an innovative approach to selectively target these malignancies. Blocking nuclear export has been proposed as a therapeutic strategy against many types of cancer based on diverse mechanisms of action, such as preventing signaling through p53 or NFκB (Mutka et al., 2009; Takeda et al., 2010). In fact, LMB was tested as an anti-cancer agent in phase I clinical trials, but due to dose-limiting toxicity, it was not pursued further (Newlands et al., 1996). Despite concerns about toxicity, targeting CRM1 remains an attractive possibility, and new, more potent and less toxic agents are being tested (Ranganathan et al., 2012).

In Chapter 1, we determined that human *CALM-AF10*-expressing leukemic cells are more sensitive to treatment with LMB than *CALM-AF10*-negative cells. Additional preclinical studies of CRM1 inhibitors in mice with *CALM-AF10* leukemias are necessary. We hypothesize that inhibition of CRM1 either alone or in combination with standard chemotherapeutics will result in regression of the leukemia. Importantly, we have discovered mechanistic explanations for why CRM1 inhibitors would prevent the growth of *CALM-AF10* leukemia cells. Elevated expression of the *Hoxa* cluster genes is considered to be a driver of leukemogenesis, and we determined that treatment with LMB quickly blunts transcription of these genes. Therefore, CRM1 inhibition directly prevents a driving mechanism of the leukemia. However, it should be noted that prolonged treatment with nuclear export inhibitors will hinder all CRM1-mediated

export, potentially resulting in toxicity. Either bolus or dose-limiting treatments may be necessary to specifically target *CALM-AF10* cells without toxic effects on normal cells.

As previously discussed, new AF10 binding partners with CRM1 interaction domains have been identified in patients with T-ALLs. Therefore, CRM1 inhibitors may have a broad therapeutic potential for patients with other types of *AF10* leukemias. We also speculate that *MLL* and *NUP98* translocation-driven leukemias may be dependent on CRM1 interaction. Thus, the therapeutic potential of CRM1 inhibitors may be broadly applicable to other leukemias as well.

In conclusion, the finding that CRM1 interaction is a critical component for *CALM-AF10*-mediated leukemogenesis is exciting for its therapeutic potential. CRM1 inhibition is a viable therapeutic target, and many CRM1 inhibitors have been characterized or are currently in development. While the specific molecular mechanisms of *CALM-AF10*-mediated leukemogenesis remain to be elucidated, we hypothesize that CRM1 inhibitors may represent a novel therapeutic approach for patients with these aggressive malignancies.

5. Materials and Methods

5.1 Generation of CALM-AF10 mutant constructs

The bicistronic MSCV-IRES-eGFP retroviral vector encoding Flag-tagged *CALM-AF10* was a kind gift from Eric Delabesse. The CALM-AF10 chimera fuses all but the last 4 aa of CALM (NCBI accession NP_001008660; also missing 8 aa from exon 17a) to exon 11 (aa 234) of AF10 (NCBI accession NP_001182555). For clarity and consistency with previous publications, CALM amino acid numbering is in reference to the longest CALM isoform (NCBI accession NP_009097.2), which is a total of 652 aa. Truncation mutants were generated by PCR amplification of *CALM* domains using primers containing a *BamHI* site and Flag tag at the 5′ end and an *NsiI* site at the 3′ end. These *CALM* products were cloned in frame with *AF10* using an intermediate plasmid in which an *NsiI* site was introduced at the 5′ end of exon 11. Point mutations within the CALM NES were generated using a Site Directed Mutagenesis kit (Agilent Technologies). NES fusion constructs were generated by cloning double stranded oligonucleotides in frame with AF10 (aa 234) using *BamHI* and *NsiI* overhangs. The CALM-AF10$^{\Delta OMLZ}$ construct was made by deleting AF10 nt 2128-2349 (coding for aa 710-783) by PCR. All constructs were verified by sequencing (Duke DNA Analysis Facility).

5.2 Infection of hematopoietic progenitors and methylcellulose assay

Mice were bred and maintained at the Duke Animal Facility. All *in vivo* and euthanasia procedures in this study were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies have been approved by the Duke University Institutional Animal Care and Use Committee (IACUC) (Protocol # A029-10-02). All efforts were made to minimize animal suffering. Primary bone marrow (BM) cells were isolated from 4-8 week old B6(Cg)-Tyr^{C-2J}/J mice (B6-albino mice, Jackson Laboratory) injected 4-5 days previously with 5-fluorouracil (150 mg/kg, tail vein injection). Hematopoietic precursor cells (HPs) were enriched from hind leg long bones by lineage depletion and were infected as previously described (Lavau et al., 1997), with the following modifications: antibodies used for lineage depletion were limited to those against CD11b, Gr1 and B220 (eBioscience). Transduced HPs were plated in methylcellulose medium (HSC-CFU media; Miltenyi Biotec) containing IL-3, IL-6, GM-CSF (all at 10 ng/ml; PeproTech), and SCF (100 ng/ml; PeproTech) at a concentration of 1,000 cells/ml/well (in a 6-well plate). Seven days later, colonies consisting of >100 cells were counted. Cells harvested from the pooled colonies were serially replated under identical conditions at a concentration of 10,000 cells/ml to generate secondary and tertiary colonies. Cytospin preparations and immunophenotypic analyses (anti-Mac-1, anti-Gr-1, anti-c-Kit antibodies from eBioscience) were performed using cells from tertiary colonies.

5.3 Cell culture

HEK293 (ATCC) and retroviral packaging Plat-E cells (Morita et al., 2000) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (Invitrogen). MEF lines were generated by immortalizing fibroblasts isolated from E14 embryos with the SV40 T/t antigen. MEFs were maintained in the same basic medium supplemented with non-essential amino acids, glutamine, and gentamicin (Invitrogen). Murine hematopoietic cells were grown in RPMI 1640 medium supplemented with 5 ng/ml murine recombinant IL-3 (PeproTech), 10% FBS, glutamine, penicillin and streptomycin. Human U937 (ATCC), P31/Fujioka (Narita et al., 1999), K562 (ATCC), and THP-1 (ATCC) cells were grown in the same medium without IL-3.

5.4 Transfection/Infection of cell lines

HEK293 and Plat-E cells were transfected by the calcium phosphate method. MEFs were infected by co-culture with filtered Plat-E supernatant in the presence of Polybrene (2 μg/ml). Murine fetal liver lines were infected by spinoculation. Transfection/infection efficiencies were verified by GFP percentage by flow cytometry (Accuri C6).

5.5 $Calm^{NULL}$ hematopoietic cell lines

CALM-deficient cells were obtained from *Picalm*^{fit1} (referred to as $Calm^{NULL}$) mice (Klebig et al., 2003; Scotland et al., 2012). $Calm^{NULL}$ *CALM-AF10* leukemias were generated by transplanting *CALM-AF10*-transduced $Calm^{NULL}$ E14 fetal liver cells into lethally

irradiated B6(Cg)-Tyr^{C-2J}/J mice. Upon development of symptoms, mice were sacrificed, and bone marrow cells were cultured with IL-3. These cells were used to detect CALM-AF10 using an anti-CALM antibody by immunofluorescence. *Calm*^{NULL} hematopoietic cell lines were also generated by *in vitro* immortalization of E14 fetal livers with *MLL-ENL* (Lavau et al., 1997). CALM-AF10 or CALM(mutNES1+2)-AF10 were ectopically expressed by retroviral transduction in these cells and their subcellular localization was assessed using an anti-CALM antibody.

5.6 Immunostaining and microscopy

Transfected HEK293 cells or infected MEFs were grown on coverslips in 6-well plates. Cells were washed with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde (20 min), permeabilized with 0.1% Triton X-100 in PBS (10 min), and blocked with 10% FBS (1 h). Cells were incubated with anti-Flag M2 (Sigma) or anti-DOT1L (Santa Cruz) for 1 hr at room temperature (RT). For IF of hematopoietic cells grown in suspension, cells were allowed to settle onto Poly-D-Lysine-coated coverslips overnight. Cells were fixed in 3.7% paraformaldehyde, permeabilized with 0.3% Triton X-100, washed, and blocked with PBS containing 12% normal donkey serum, BSA, and Tween-20 for 1 hr. Because of the lower expression of the fusion protein in these hematopoietic cells, CALM-AF10 could not be detected using the anti-Flag antibody, and a polyclonal anti-CALM antibody (Sigma) was used. This antibody also detects endogenous murine Calm, and therefore we performed these experiments in *Calm*^{NULL}

CALM-AF10-expressing cells. All cells were incubated with AlexaFluor568 or 488-conjugated secondary antibodies (Invitrogen) for 40 min at RT. After further washing, cells were stained with DAPI and mounted onto slides using Fluoromount-G (Southern Biotech). All confocal images were obtained at the Duke Light Microscopy Core. Samples were examined at RT using a Zeiss LSM 510 confocal on an Axio Observer microscope using a Zeiss Plan-Apochromat 63x/1.4 or a 100x/1.4 oil objective. Pinholes were set to 1 airy unit for each channel, line averaging of 8, 1024 x 1024 image format, and a 2X optical zoom for the 100x objective. Images of cell and colony morphology were obtained with the Leica Microsystems DFC425 attached to a Leica DMLB at RT.

5.7 Bone marrow transplantation and assessment of mice

Recipient B6-albino mice were lethally irradiated (10 Gy in two split doses, 3 hr apart) using an X-RAD 320 irradiator 24 hr prior to transplantation. Mice were injected (tail vein) with 50,000-200,000 infected, lineage depleted HPs [CALM-AF10, NES^{CALM}-AF10, CALM(520-583)-AF10, or Hoxa9/Meis1 (Morgado et al., 2007)] along with 200,000 freshly harvested bone marrow cells to ensure radioprotection. Hematopoietic engraftment of GFP-positive cells was assessed by flow cytometry of peripheral blood leukocytes. Mice were monitored for signs of disease and sacrificed when moribund by CO_2 euthanasia. Spleens were weighed, and morphology of leukemia cells was analyzed by peripheral blood and bone marrow smears using the Diff-Quik Stain (Dade Behring, Inc). The concentration of leukocytes in the blood was determined by flow cytometry

(Accuri C6) following lysis of red cells with ammonium chloride solution (StemCell Technologies). The percentage of blasts (GFP-positive) and bone marrow immunophenotype (all antibodies from eBioscience) were analyzed by flow cytometry (Accuri C6).

5.8 Western blotting

Western blots were performed according to standard protocols. Primary antibodies included: Flag M2 (Sigma), CALM (Sigma), Actin (Sigma), Histone H3 (Cell Signaling), and di-methylated H3K79 (Cell Signaling). Fluorescently conjugated secondary antibodies were incubated for 1 hr at RT, and blots were developed using the Odyssey Infra-red imaging system (Li-Cor Biosciences). To quantitate levels of di-me-H3K79, the integrated intensities of the bands were measured by the Odyssey imaging system and normalized to Actin or total Histone H3.

5.9 Real Time RT-PCR of Hoxa genes and RT-PCR of CALM-AF10

Total RNA was isolated from MEFs or leukemic cells using the RNeasy Mini kit (Qiagen). Total RNA was reverse transcribed using the Superscript II kit (Invitrogen). Quantitative PCR amplification was performed using the iQ Sybr Mix (Bio-Rad) with the iQ5 Optical System (Bio-Rad). Expression levels were normalized to the levels of the endogenous housekeeping genes *β2M* and *GAPDH* by the comparative threshold (CT) method. Primers used for Real Time RT-PCR are listed in Table 1.

Table 2: Primers for Real Time PCR

Gene	Forward (5'-3')	Reverse (5'-3')
B2M	ACCGGCCTGTATGCTATCCAGAAA	GGTGAATTCAGTGTGAGCCAGGAT
GAPDH	CCTGGAGAAACCTGCCAAGTATG	AGAGTGGGAGTTGCTGTTGAAGTC
HOXA5	CTCATTTTGCGGTCGCTATCC	AGATCCATGCCATTGTAGCC
HOXA7	TATGTGAACGCGCTTTTTAGCA	GAAGTCGGCTCGGCATTTTG
HOXA9	CCCCGACTTCAGTCCTTGC	GATGCACGTAGGGGTGGTG
HOXA10	GGAAGCATGGACATTCAGGT	CCAGGCAAGCAAGACCTTAG
HOXA11	CCCCTGGTGGTTCACTCTTA	CTTGGGGCACACAGTTTCTT

5.10 Chromatin Immunoprecipitation (ChIP):

Modification-specific histone ChIP assays were performed as described previously (Okada et al., 2005). Immunoprecipitation was performed with 1 μg of anti-H3K79me2 or anti-H3K4me3 antibodies (Abcam) incubated overnight at 4°C. Salmon sperm-conjugated protein G sepharose beads (35 μL; Millipore) were then added and rocked for an additional 3 hr at 4°C. Following RNAse A and proteinase K treatment, DNA was purified with a PCR purification kit (Qiagen) and amplified by Real Time PCR. Amplification values are normalized to input and are presented as fold enrichment over negative control (empty vector or Hoxa9/Meis1). Primer sequences used to amplify regions of the *Hoxa* genes are listed in Table 3.

Table 3: Primers for Chromatin Immunoprecipitation

Amplicon	Forward (5'-3')	Reverse (5'-3')
Hoxa5	CTCCACCCAACTCCCCCATT	GGACATGTACTCGGTTCCCT
Hoxa7	AACCCTTCCCCTAAACGCCTC	AAAAGGTCGCCAGTCTTCCAG
Hoxa9	ATCTGTATGCCTAGTCCCGCTCC	TTGATGTTGACTGGCGATTTTC
Hoxa10	GGCTAGACTCACTCTGGCAA	GCAGGGAAGCTCGGTTTAGG
Hoxa11	GGAAGCAACAGATCGTCACTCG	TGAGTTACACCGGCGATTACG

5.11 Inhibition of CRM1-dependent nuclear export

Prior to IF analysis, HEK293 and murine leukemia cells were treated with 10 nM or 0.1 nM LMB for 1 h or 12 hr, respectively. MEFs were treated with 1 nM LMB for approximately 24 hr before di-me H3K79 ChIP and with 0.7 nM LMB for 2.5 hr before Flag ChIP analysis. Human leukemic cell lines (U937, P31/Fujioka, K562, and THP-1) were grown in the presence of varying concentrations (0, 0.1, 0.5, and 1 nM) of LMB (Sigma) for 17 and 42 hours. Cell viability was analyzed by flow cytometry, and total cell number was recorded as a percent of untreated.

5.12 Statistical Analysis

Data are presented as mean plus or minus SEM (n = 3 or more). Statistical analysis was performed by one-way ANOVA followed by Dunnett's post-test when three or more groups of data were compared. Statistical analyses of cell viability experiments were performed by two-way ANOVA followed by Bonferroni's Multiple Comparison Test.

Values were considered statistically significant when *P* values were less than .05. All analyses were done using PRISM software (GraphPad Software, Inc.).

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Book Chapters

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